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Behavioural Flexibility in Bumblebees (Bombus impatiens)

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Abstract

Foraging bumblebees (*Bombus impatiens*) extract nectar and pollen from a wide variety of morphologically distinct flower species, referred to as flower handling. Bumblebees learn this behaviour and acquisition of multiple flower handling techniques is a demonstration of behavioural flexibility. The purpose of this thesis is to understand how bumblebees are able to forage flexibly. This research has three specific goals: (1) to identify the cognitive mechanisms that support flower handling learning, (2) to understand how bumblebees avoid interference costs between multiple handling techniques, and (3) to explore the relation between behavioural flexibility and the mushroom bodies of the bumblebee brain. To address the first two goals, I developed a laboratory model of flower handling. The model consisted of a tube with a plastic door insert that bumblebees moved to access a nectar reward. The door was designed to be similar to a flower petal that a bee would lift to access a nectary in a real flower. All bees demonstrated the same set of motor behaviours and showed improvement across trials by increasing the frequency with which they used the successful behaviour. The apparatus was then adapted to measure bees' ability to switch between two handling tasks, representing two different flower morphologies. Two variations of the apparatus were used, each of which required a different innate motor pattern for successful removal of the door. Bees switched between the two tasks by changing only the frequency that they engaged in each successful motor behaviour. The role of the mushroom bodies in behavioural flexibility was examined by training bees on a measure of behavioural flexibility, reversal learning, and relating performance to volume of the mushroom bodies and their components. Performance on the reversal task did not correlate with mushroom body volume. My overall findings are that bumblebees use a combination of innate motor patterns and learned associations to forage on a variety of flower species and the

flexibility of individual bumblebees is not related to individual variation in volume of the mushroom bodies and their components.

Keywords

bumblebees, behavioural flexibility, flower handling, reversal learning, mushroom bodies

Co-Authorship Statement

All data chapters are co-authored with Dr. David Sherry. Dr. Sherry provided funding for all experiments, contributed to experimental design, provided input on statistical analysis, and edited all chapters.

Chapter 2 will be submitted for publication. Dr. David Sherry and Jordan Phelps will be co-authors. Jordan Phelps contributed to data collection, initial analysis of video recordings, and development of the video analysis techniques.

Chapter 3 will be submitted for publication. Dr. David Sherry will be a co-author.

Chapter 4 will be submitted for publication. Dr. David Sherry will be a co-author.

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List of Abbreviations

AL: antennal lobes

Ca: calyces

CB: central body

DMTS: delayed matching-to-sample

HIREC: human-induced rapid environmental change

ITIs: inter-trial intervals

La: lamina

Lo: lobula

MAB: Multi Access Box

MB: The mushroom bodies

Me: medulla

PE: proboscis extension

PER: proboscis extension reflex

S+: rewarded stimulus

S-: unrewarded stimulus

SOG: suboesophageal gangli

Chapter 1

1 Introduction

In recent decades awareness of the importance of bees and other pollinators to our food supply and economy has made them the focus of conservation efforts (Byrne & Fitzpatrick, 2009). Bees have also been at the forefront of research in animal cognition due to surprising findings on bees' success on complex cognitive tasks (Giurfa, 2015; Perry, Barron, & Chittka, 2017). For these reasons continued investigation of learning and memory in bees is important. This is particularly true of research that links cognition and conservation and aims to understand the cognitive processes underlying the natural behaviour of bees in their role as pollinators.

The research described in this thesis focuses on behavioural flexibility in bumblebees. Behavioural flexibility describes how animals adapt to changes in their environment and may be informative for predicting both bees' successes and exposures to risk caused by human-induced rapid environmental change (Sih, Ferrari, and Harris, 2010). Measures of behavioural flexibility have historically been useful in examining the mechanisms of learning and memory (Mackintosh, 1969; Bitterman, 1969; Davey, 1989; Shettleworth, 1998; 2010). In the subsequent chapters I describe a series of experiments on the foraging behaviour of bumblebees, focusing on the cognitive and neural mechanisms that support those behaviours.

In this chapter, I provide background information on behavioural flexibility, foraging, learning by bees, the bee brain, and an introduction to my study species *Bombus impatiens.*

1.1 Behavioural Flexibility

The generally accepted definition of behavioural flexibility is an animal's ability to respond to changes in its environment (Ragozzino, 2007; Coppens et al., 2010). The use of such a broad definition has resulted in the topic of behavioural flexibility becoming a vast minefield

of different definitions, techniques, and applications that spans fields from neuroscience, to animal cognition, to field biology and everything in between (Audet & Lefebvre, 2017). Given the vastness of the field, I have grouped considerations of behavioural flexibility into proximate questions (Tinbergen, 1963), focusing on cognitive mechanisms, and ultimate questions (Tinbergen, 1963), focusing on the adaptive value of behavioural flexibility and its implications for reproductive success.

1.1.1 Cognitive mechanisms of flexibility

Much of the research on the cognitive mechanisms underlying behavioural flexibility has revolved around its use as a measure of general animal intelligence (Reader & Laland, 2002; Roth & Dicke, 2005; Reader, Hager, & Laland, 2011). The driving force behind of many of these studies has been to find a behavioural measure that correlates with brain volume (Reader & Laland, 2002; Roth & Dicke, 2005; Overington et al., 2009). The role of behavioural flexibility in understanding intelligence has been approached in two similar but slightly different ways. In the first, researchers take the consistently high performance by a particular species or taxon on a variety of tasks, that is their ability to flexibly apply a general cognitive skill set, as evidence of general intelligence (Reader et al., 2011). In this way behavioural flexibility is seen as a consequence of *g*, or general intelligence (Reader et al., 2011). In the second approach, animals are tested on a specific task for behaviour that the researchers have decided measures behavioural flexibility and better performance is regarded as more intelligent or cognitively complex. In this way, behaviour flexibility becomes the operationalized definition of intelligence (Lefebvre, Reader, & Sol, 2004). Frequently used measures of behavioural flexibility are puzzle box or other problem solving (Webster & Lefebvre, 2001; Auersperg et al., 2011; Auersperg, Gajdon, & von Bayern, 2012), foraging innovation (Sol & Lefebvre, 2000;

Lefebvre et al., 2004; Overington et al., 2009), and reversal learning (Bond, Kamil, & Balda, 2007). It is often the case that animals are assessed on more than one of these tasks to create a general profile of the species' behavioural flexibility and that profile is equated with intelligence (Tebbich, Sterelny, & Teschke, 2010).

Although studies of behavioural flexibility undeniably provide interesting data on the behaviour of a wide variety of species, the loose definition of behavioural flexibility and inconsistencies in measuring behavioural flexibility are problematic (Auersperg et al., 2012; Audet & Lefebvre, 2017). The frequently used assessments of behavioural flexibility, problem solving, foraging innovations and reversal learning, are often assumed to measure the same construct, but that may not be the case. In a reversal task an animal acquires an initial discrimination with a rewarded stimulus (S+) and an unrewarded stimulus (S-) and then the reward contingencies are reversed so that the original S+ becomes the S- and the original S- becomes the S+ (Shettleworth, 1998/2010). The task can then be expanded to include repeated reversals, called a serial reversal task. In reversal tasks an animal is successful when it is able to inhibit responding to a previously rewarded stimulus and does not perseverate in the absence of reward (Shettleworth, 1998/2010). In problem solving, however, task success is often correlated with an animal's continued attempts to solve a task despite not receiving a reward, that is its perseveration (Audet, Ducatex, & Lefebvre, 2016). Consequently, continuing to produce a behaviour in the absence of reward results in poor flexibility as measured on reversal tasks, but can result in high flexibility when measured using problem solving tasks. Using field assessment of foraging innovations as a measure of behavioural flexibility also comes with numerous potential problems. Innovations can be defined as 'a qualitative break with species- or population-typical behaviour' (Greenberg, 2003), but that definition can be difficult to

operationalize in field observations. Additionally, the use of innovation rate as a measure of flexibility usually relies not on firsthand accounts, but on surveys of accounts from multiple field reports, making it difficult to assess the consistency with which operationalized definitions have been applied (Greenberg, 2003).

Behavioural flexibility has been a popular topic of study in animal cognition and that trend is likely to persist given how fruitful work on the topic has been in characterizing animal behaviour and relating behaviour to brain evolution. However, it is essential to move from vague definitions of behavioural flexibility to a recognition that numerous cognitive mechanism can generate flexible performance on cognitive tasks and that some of those mechanisms may be simple (Audet & Lefebvre, 2017). The need for greater care in experimental techniques for studying behavioural flexibility has been acknowledged by researchers in the field and has even resulted in novel techniques intended to resolve some of the challenges of studying behavioural flexibility (Auersperg et al., 2012). Auersberg et al. (2012) developed a puzzle box to be used for cross species comparisons of behavioural flexibility (Figure 1-1). The puzzle box takes into account the morphological differences that exist between species and allows for multiple different solutions which may prevent cross-species comparisons from being biased towards a particular species. The puzzle box has been validated in kea and New Caledonian crows, demonstrating successful use of the same apparatus with multiple species (Auersberg et al., 2012). Although the cross-species puzzle box may not develop into the standard apparatus for studying behavioural flexibility, given its reliance on object manipulation and absence of clear ecological relevance for most species, it acknowledges the challenges of studying behavioural flexibility.

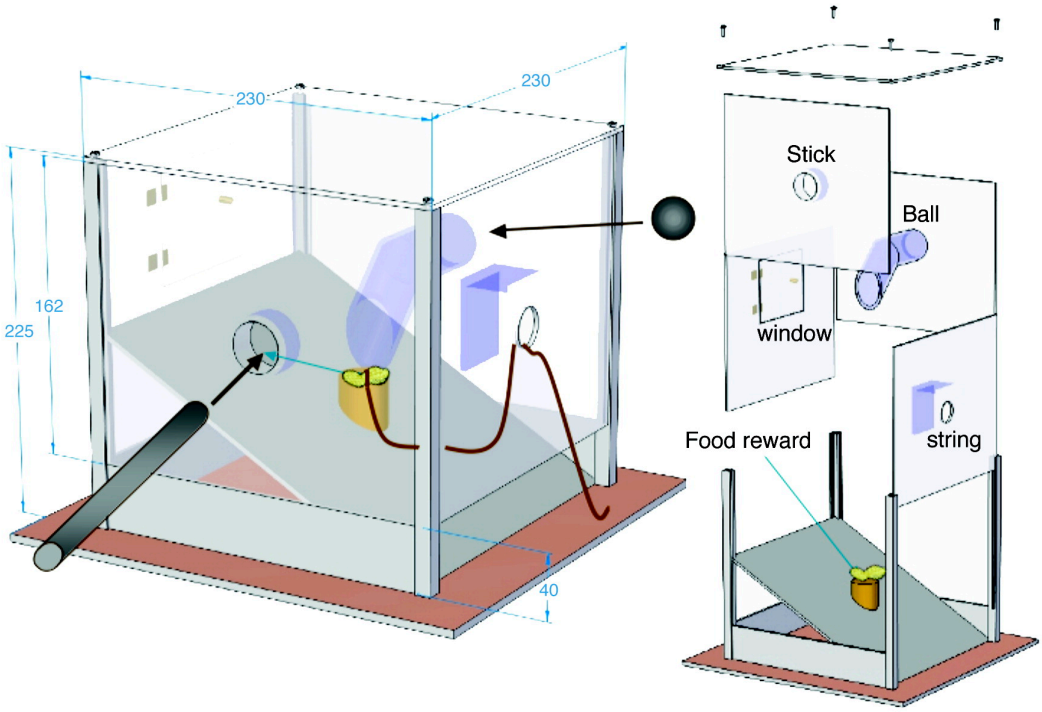

Figure 1-1 The Multi Access Box (MAB) designed for use in comparative studies of behavioural flexibility with multiple species. The box has four different solutions that provide access to the food reward: (1) pulling a string accessible from outside of the apparatus, (2) opening a window at the back of the apparatus gaining access to the reward, (3) inserting a stick into the apparatus to push the food reward out of the apparatus, and (4) dropping a ball into the apparatus to knock the food reward off the platform and out of the apparatus. (Figure from Auersperg et al., 2012)

1.1.2 Adaptive value of flexibility

Research on behavioural flexibility is not limited to considerations of cognition, it has also gained importance regarding ultimate questions about adaptation and evolution. In a study of the success of avian species following introduction to New Zealand, Sol and Lefebvre (2000) found that species which showed more foraging innovations were more successful in their new environment. This relationship between foraging innovations and invasion success was confirmed by a follow up study examining the success of avian species following introduction to different regions world wide, showing that the original finding was not region specific (Sol, Timmermans, & Lefebvre, 2002). These studies suggest that behavioural flexibility can be

adaptive in situations where a species must exploit a new environment. Behavioural flexibility has also been suggested to play a role in adaptive radiation, that is phenotypical divergence of species from a single lineage (Schluter, 2000). Tebbich et al. (2010) examined the relationship between behavioural flexibility and one of the most famous examples of adaptive radiation, Darwin's finches in the Galapagos. The hypothesis was that the finches possessed a high degree of behavioural flexibility upon arriving in the Galapagos, resulting in high rates of innovation and consequent phenotypic variation in foraging. This acquired phenotypic variation in foraging changed the selective pressures different finches were exposed to and led eventually to speciation. A battery of behavioural flexibility tests administered to three species of Darwin's finches showed a high rate of behavioural flexibility in all species and supported the role of behavioural flexibility in the invasion success and adaptive radiation of the Galapagos finches (Tebbich et al., 2010). The importance of behavioural flexibility is not limited to invasion success but is also applicable to survival under conditions of human-induced rapid environmental change (HIREC). Sih et al. (2010) conducted a meta-analysis on the characteristics that predict the success of species in response to HIREC and found that behavioural flexibility was a key component in their model.

1.2 Specialist and generalist foraging strategies

Although individual bumblebees often show flower constancy (a tendency to visit a particular flower species), bumblebee species are typically generalist foragers (Heinrich 1979/2004). This foraging style is in contrast to that of specialist bumblebee species that forage exclusively on a single flower type (Laverty & Plowright, 1988). Specialist species are repeatedly faced with the same predictable foraging challenges both within the life of individuals and across generations, consequently specialist species sometimes possess behavioural or

morphological adaptations to those challenges (Drummon, 1983; Thøstesen & Olesen, 1996; Yamada & Boulding, 1998; Laverty & Plowright, 1988; Goulson & Darvill 2004). For generalists, the overall challenge of obtaining resources is the same, but the particulars of how they obtain those resource (which flowers they extract nectar from) can vary among individuals and over generations.

What advantage does a specialist obtain in exchange for reducing the range of resources that it can exploit? Comparisons of specialist and generalist foragers in a variety of taxa show that specialists are more efficient at their foraging specialization than generalists are (Drummond, 1983; Laverty & Plowright, 1988; Thøstesen & Olesen, 1996; Yamada & Boulding, 1998). How do specialists become more efficient? There are two possibilities: (1) they have evolved morphological features that facilitate exploiting a particular resource, or (2) they have evolved behaviour that facilitates exploiting a particular resource (Laverty & Plowright, 1988). Examples of morphological differences include the increased claw strength of crab species that specialize on hard-shelled prey compared to omnivorous generalist crabs (Yamada & Boulding, 1998), and the relationship between tongue length and floral specialization in bumblebees (Goulson & Darvill 2004). Alternatively, specialization can occur at the behavioural level. A comparison of aquatic specialist snakes and aquatic-terrestrial generalists found that specialists were behaviourally more efficient at capturing aquatic prey compared to the generalist species (Drummond, 1983). Specialist bumblebees, *Bombus consobrinus*, that have a longer proboscis than generalist bumblebees (Thøstesen & Olesen, 1996; Kearns & Thomson, 2004) also show behavioural differences (Laverty & Plowright, 1988). Compared to a generalist species of bumblebee, the specialists were able to locate and probe the nectary of their speciality plant monkshood (*Aconitum*) more efficiently (Laverty & Plowright, 1988).

In the absence of solid empirical evidence, it has often been assumed generalist behavioural foraging strategies and behavioural flexibility occur together. Studies directly quantifying the relationship between generalist foraging and rates of innovation, however, have shown the relationship to be somewhat nuanced (Overington et al., 2011). Overington et al. (2011) compared innovation rate to ecological generalism in 193 bird species and found that habitat generalism (the ability to survive in different habitat types) correlated with innovation rate, but that diet generalism (foraging on a wide variety of resources) did not. A much expanded investigation of generalism and innovation, however, that included 765 bird species did find a relationship between diet generalism and innovation (Ducatez, Clavel, & Lefebvre, 2015). Diet generalism correlated with food type innovation, in which birds exploited novel food types, and technical innovations, in which birds generated novel techniques for handling previously exploited resources. There does seem to be a positive relationship between foraging generalism and behavioural flexibility, but inconsistencies in research findings on the topic demonstrate the importance of establishing the type and mechanism of a generalist foraging strategy when using it as an indication of behavioural flexibility (Ducatez et al., 2015).

1.3 Learning in Bees

Research on learning in bees dates to the Nobel Prize winning work of von Frisch (1967). The early studies done by von Frisch (1967) demonstrated that bees can discriminate between colours and can pair colours with rewards. This foundational work was the starting point for extensive investigations on associative learning in bees (Menzel, 1990). Among the most important techniques for research on simple learning processes in bees is the paradigm to study proboscis extension reflex (PER; Takeda, 1961; Giurfa & Sandoz, 2012). In this paradigm, stimuli - usually olfactory - are presented to harnessed bees and paired with sucrose reward

which triggers an unconditioned response (Bitterman et al., 1983). This classical conditioning paradigm became a core technique in bee learning studies and its use led to extensive understanding of the neural circuit responsible for olfactory conditioning in the bee brain (Giurfa & Sandoz, 2012).

Although research on the basic aspects of learning and memory in bees has a long history, research on more complex cognitive processes is a more recent development. Zhang, Bartsch, and Srinivasan (1996) trained honeybees to navigate through a maze using visual cues. The initial maze design was then further adapted into a Y-shaped design that consisted of a series of connected cylinders (Zhang, Lehrer, & Srinivasan, 1999; Figure 1-2). Despite the differences between the maze used for bees and traditional operant testing boxes used for matching tasks with laboratory animals such as pigeons (Roberts, Strang, & Macpherson, 2015), the 'Y-maze' design allowed researchers to conduct delayed matching-to-sample (DMTS) testing in honeybees. In the Zhang et al. (1999) maze bees enter an initial chamber in the apparatus and view a sample stimulus. They then travel through the maze to a choice chamber where they can follow either the matching stimulus or the non-matching stimulus. A choice of the matching stimulus results in bees entering a chamber that contains a sucrose reward. DMTS paradigms are the foundation for a wide variety of cognitive testing, so use of the paradigm allowed researchers to demonstrate honeybees' ability to discriminate based on numerosity (Gross et al., 2009) and learn concepts (Giurfa et al., 2001), and even show some evidence of metacognition through adapting the y-maze design to be operationally similar to the Hampton (2001) metamemory paradigm (Perry & Barron, 2013).

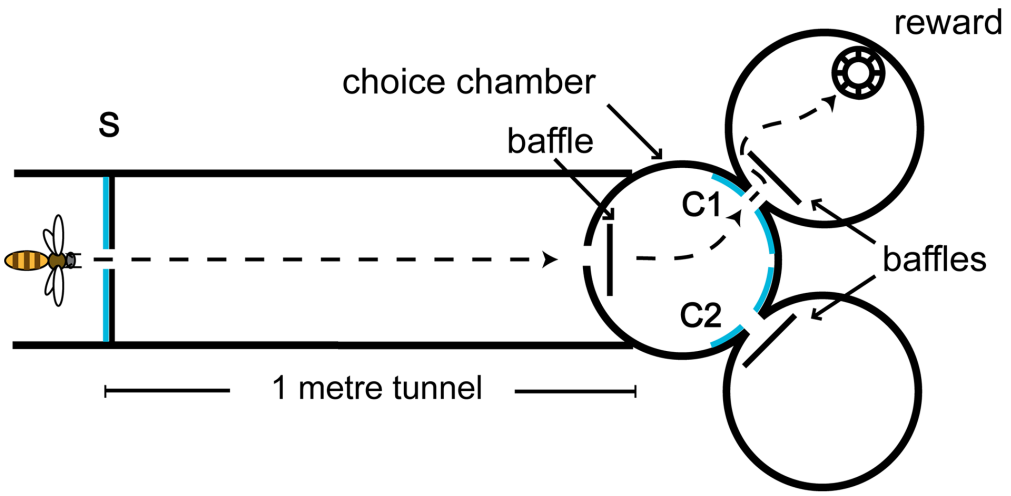

Figure 1-2 The Y-maze apparatus as used for DMTS experiments with honeybees. The bee enters the apparatus and is presented with a sample stimulus (S). The bee then continues through the apparatus for a distance that generates a delay until it reaches the choice chamber. In the choice chamber the bee encounters two choice stimuli (C1 and C2), one of which matches the sample stimulus and one that does not. Entering the reward chamber indicated by the matching stimulus results in a sucrose reward. The entrances to the choice and reward chambers are covered by baffles that prevent the bee from seeing the choice stimuli or reward before entering the chamber. (Figure from Zhang et al., 1999)

Bumblebees also have demonstrated impressive learning abilities. Bumblebees are able to navigate a T-maze by using a colour cue (Chittka & Thomson, 1997). They failed, however, to learn the Y-maze DMTS task described in Figure 1-2 for honeybees (Sherry & Strang, 2015). Bumblebees have successfully learned a different DMTS task, however, in which the apparatus was a modified version of the radial arm maze developed for rats by Olton and Samuelson (1976). Research on DMTS in bumblebees suggests that they are capable of using matching to solve a task, like honeybees do, but are predisposed to use alternative strategies if possible (Thompson & Plowright, 2016). More recently bumblebees have been the subject for investigation of problem solving and physical cognition (Mirwan & Kevan, 2014; Alem et al., 2016; Loukola, Perry, & Chittka, 2017). The apparatuses that have been used in this research are modelled after those used to study physical cognition in birds and primates (Heinrich & Bugnyar, 2005; Schmitt, Pankau, & Fischer, 2011). These tasks have required bumblebees to lift

and roll objects (Mirwan & Kevan, 2014; Loukola et al., 2017) and pull strings (Alem et al., 2016), all of which were successfully completed by the bumblebees. In one of the physical cognition experiments with bumblebees, the successful solution spread socially within colonies, leading the authors to suggest bees were demonstrating rudimentary culture (Alem et al., 2016).

It is clear that bees have a rich behavioural repertoire, including impressive learning abilities in a variety of domains, and are not the 'reflex machines' that they were once believed to be (Menzel, 1990).

1.4 The bee brain

All of the learning described in the previous section was accomplished by bees with a brain that contains around 1 million neurons (Chittka & Niven, 2011), in contrast to the approximately 200 million neurons in the brain of the more traditional subject of animal learning cognition research, the laboratory rat (*Rattus norvegicus*; Herculano-Houzel & Lent, 2005). In addition to their learning abilities, bees have a large repertoire of behaviour. Chittka & Niven (2011) identified 59 distinct behaviours of honeybees, which is perhaps small compared to laboratory rats, but not smaller by a factor of 200 million as is the case with neuron number. How is it that bees are able to generate so many behaviours with so few neurons? If this question is rephrased to ask not why bee brains are so small, but why vertebrate brains are so large, then the answer lies largely in the relationship between brain and body size (Striedter, 2005; Roth & Dicke, 2005). Larger organisms take in more sensory input than smaller organisms and require more neural architecture to process that information (Striedter, 2005). Additionally, larger organisms require a nervous system that can generate large scale motor output (Striedter, 2005). The scaling of brains to body size can account for much of the discrepancy in brain size between bees and vertebrates, but even taking this into consideration the bee brain still seems to generate

remarkable behavioural output given its size (Chittka & Niven, 2011). Having been dubbed 'the amazing mini-brain' (Giurfa, 2003), the bee brain is of interest to neuroscientists investigating the interplay of brain and behaviour. The simplicity of the bee brain provides an opportunity to map behaviour to neural structures and circuits in a way that is not presently possible in larger brains (Heisenberg, 1998).

1.4.1 Structure

The bee brain has three separate divisions, the *protocerebrum*, the *deutocerebrum*, and the *tritocerebrum* (Snodgrass, 1910; Figure 1-3). The *deutocerebrum* includes the antennal lobes, which are the olfactory sensory region of the bee brain. The *tritocerebrum* controls the sympathetic system. The *protocerebrum* is the division of particular interest here because it includes the mushroom bodies (*corpora pedunculata*), the brain region associated with learning in insects. In addition to containing the mushroom bodies the *protocerebrum* includes the optic lobes, which are the visual sensory region, and the central complex, which controls motor output (Farhbach, 2006). The structure of the mushroom bodies varies dramatically in size and shape across arthropod taxa, but in all instances they consist of Kenyon cells (Fahrbach, 2006), named for F. C. Kenyon the researcher who first described them in 1896 (Fahrbach, 2006). Kenyon cells appear bilaterally and consist of a neurite with axon-like and dendrite-like branches (Fahrbach, 2006). Developmentally, the dendritic branch of the Kenyon cells undergoes arborization that produces the calyces of the mushroom bodies, and the axon-like branch generates the two mushroom body lobes (Fahrbach, 2006). There are three different types of Kenyons cells in the bee brain, varying in size and location, that are the result of neurogenesis patterns during development (Fahrbach, 2006). The calyces vary in the insect brain from absence in dragonflies,

to doubled calyces, a medial and lateral calyx in each hemisphere, found in Hymenoptera (Fahrbach, 2006).

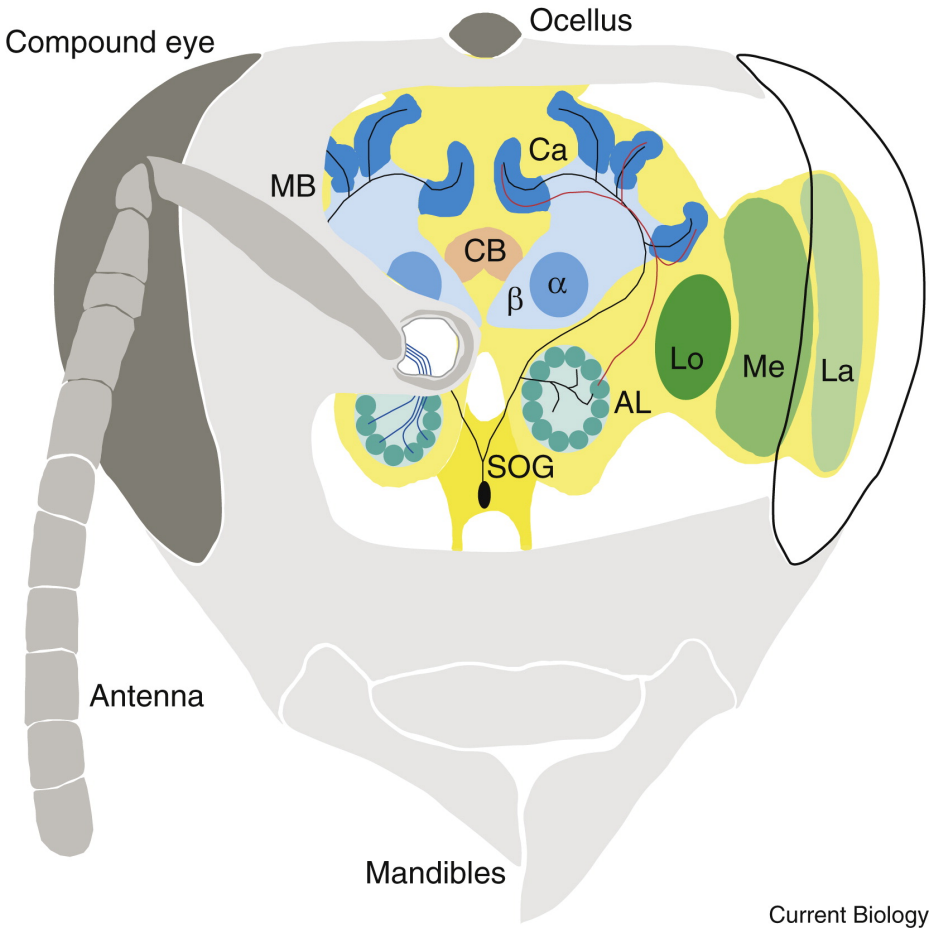

Figure 1-3 The major structures of the bee brain and their location within the head capsule. The mushroom bodies (MB) of the *protocerebrum* are shown in blue, with the calyces (Ca) in dark blue, and the peduncle and lobes shown in light blue. The central body (CB) is also part of the *protocerebrum* as are the optic lobes shown in green, the lobula (Lo), the medulla (Me) and the lamina (La). The olfactory regions of the *deutocerebrum* are the antennal lobes (AL). The suboesophageal ganglion (SOG), shown just below the antennal lobes, innervates lower parts of the head, and plays a role in olfactory conditioning. (Figure from Chittka and Niven, 2009).

Aside from gross morphological differences between taxa, the internal structure of the calyces also varies across taxa (Strausfeld et al., 1998). In the majority of insects the primary input into the calyces is olfactory but in Hymenoptera a considerable amount of input also comes from visual regions (Strausfeld et al., 1998). These inputs from olfactory and visual regions are

segregated with the calyces. The lip region receives olfactory input, the collar receives visual input, and the basal ring receives input from both visual and olfactory sensory regions, though the zone of input within the basal ring differs for each modality (Strausfeld et al., 1998; Fahrbach, 2006). The presence of projections from both visual and olfactory sensory to the mushroom bodies makes the mushroom bodies a multimodal sensory integration centre in the bee brain (Strausfeld et al., 1998).

1.4.2 Mushroom body evolution and function

The large variation in mushroom body morphology across insect taxa provides an exciting opportunity for investigations of the evolutionary history and function of the mushroom bodies (Strausfeld et al., 1998). Research on these questions began shortly after Dujardin (1850) discovered the mushroom bodies when he conducted experiments on their function by decapitating different species on insect that differed in the size of their mushroom bodies. He observed that those insects with smaller mushroom bodies displayed greater muscle coordination following decapitation and concluded that the size of the mushroom bodies was indicative of free will, with small mushroom bodies providing automatic or innate behaviour and large mushroom bodies providing greater behavioural control (Dujardin, 1850; Strausfeld et al., 1998). Although Dujardin's conclusions did not refer specifically to learning and memory, he hypothesized that the size and complexity of the mushroom bodies was correlated with complexity of behavioural output and intelligence (Strausfeld et al., 1998). Clear evidence for a relationship between learning and the mushroom bodies would come considerably after Dujardin's work in the ablation studies of de Belle & Heisenberg (1994).

The association between mushroom bodies and behavioural complexity and learning, suggests that the increase in mushroom body volume, particularly in Hymenoptera, is due to

evolutionary pressure for greater learning capacity (Farris & Roberts, 2005). This is further supported by two lines of research, the first a between species comparison and the second a within species comparison between different castes of bees. Comparisons of generalist foraging insect species to specialist foraging species show that gyri-like structure of the calyces, found in the Hymenoptera, occurs only in generalist species (Farris & Roberts, 2005). The gyri-like calyces have larger surface area and volume, suggesting that expansion of the calyces is required to support the increased flexibility and learning demands of generalist foraging (Farris & Roberts, 2005). This conclusion from phylogenetic comparisons is supported by research showing that the mushroom bodies of foraging honeybees and bumblebees are larger than bees that are engaged in tasks within the hive (Farris, Robinson, & Fahrbach, 2001; O'Donnell, Donlan, and Jones, 2004). These results support a relationship between the learning demands of foraging and expansion of the mushroom bodies. There are competing theories however on the driving force behind both developmental and evolutionary expansion of the mushroom bodies. Molina and O'Donnell (2007) found that developmental changes in a eusocial wasp occurred not coincident with foraging onset, but with social dominance. The relationship between larger mushroom bodies and social factors also has evolutionary support given that the mushroom bodies in Hymenoptera, an order containing eusocial insects, are large and contain gyri-like calyces (Strausfeld et al., 1998). Additionally, comparative work in social wasps found that the increased social interaction demands on queens is related to mushroom body volume and not the foraging demands on workers (O'Donnell, Clifford, and Molina, 2011). Regardless of whether mushroom body expansion in bees is due to increased foraging complexity or increased social complexity the relationship between mushroom body volume and behavioural complexity is generally supported.

1.5 Study species

All experiments described in the following chapters were conducted with *Bombus impatiens* (Figure 1-4). *Bombus impatiens* is a bumblebees species in the subgenus *Pyrobombus* that is native to Ontario and a large part of the Eastern United States (Kearns & Thomson, 2001). A survey of North American bumblebee species showed *Bombus impatiens* to be the most prevalent in eastern regions (Cameron et al., 2011). *Bombus impatiens* nests below ground, is a short tongued species, and a generalist forager (Kearns & Thomson, 2001). *Bombus impatiens* shows the typical social structure and division of labour of bumblebees, consisting of a queen, female workers, and males produced during the reproductive phase of the annual colony cycle (Heinrich 1979/2004; Kearns & Thomson,2001). The colony cycle includes the emergence of queens from hibernation in the spring, the production of workers throughout the summer, and the production of reproductive queens and males at the end of the cycle (Kearns & Thomson, 2001). During the first phase of the colony cycle the queen both lays eggs and forages to provision the colony. Following the development of the first batch of workers the queen remains in the colony to produce eggs and the female workers take over provisioning the colony (Heinrich 1979/2004; Kearns & Thomson, 2001). Worker bumblebees are largely non-reproductive, though they can lay unfertilized eggs, and they complete a variety of tasks within the nest as well as foraging to collect nectar and pollen for the colony. Bumblebees do not show the age polyethism that is typical of honeybees, wherein a worker will complete tasks within the hive for the first couple of weeks of life and then transition to foraging (Robinson, 1992). In bumblebees division of labour appears to be determined by size, with larger workers engaging in foraging tasks and smaller workers remaining in the colony (Goulson et al., 2002).

Figure 1-4 Bombus impatiens forager engaged in a colour discrimination.

Bombus impatiens have been used extensively as both commercial pollinators and research subjects. *Bombus impatiens* pollinate a variety of commercial species (Artz & Nault, 2011), but are most important in the production of tomatoes (Velthuis & van Doorn, 2006). Their importance to tomato production is due to sonication, in which a bumblebee grasps a flower and vibrates its body, triggering the release of pollen. Tomato flowers require this process to release pollen, so in the absence of bumblebees tomato flowers must be mechanically vibrated (Velthuis & van Doorn, 2006). Along with their commercial availability *Bombus impatiens* is growing in popularity as a research subject due to their behavioural repertoire, success on a variety of learning and memory tasks, and their potential for neuroscience research (Riveros and Groneberg, 2009).

As an important wild pollinator, an important commercial pollinator, and a species for which there is previous experimental research on learning and cognition, *Bombus impatiens* is an ideal subject to explore the topics of interest in this thesis.

1.6 Dissertation structure

The overall objective of this thesis is to increase our understanding of the cognitive and neural mechanisms of the flexible behavioural output of bumblebees. This objective was divided into three specific goals that correspond to the three data chapters.

In Chapter 2 I describe the process of developing and validating a model for studying flower handling, the process of extracting nectar from flowers. The model is then used to generate a detailed characterization of the learning component of flower handling and propose cognitive mechanisms for this behaviour.

Chapter 3 uses the model developed in Chapter 2 to revisit Darwin's interference hypothesis (Darwin, 1876). The hypothesis posits that switching between flower types while foraging should result in efficiency costs. The hypothesis has not been supported by empirical findings (Woodward & Laverty, 1992) and the goal of this chapter is to determine why the predicted efficiency costs seem not to occur.

The final data chapter, Chapter 4, explores the relationship between behavioural flexibility and the mushroom bodies of the bumblebee brain. Previous research has confirmed a relationship between mushroom body volume and learning (Gronenberg & Couvillon, 2010), but not behavioural flexibility. Chapter 4 describes an experiment in which the correlation between bumblebees' performance on a test of behavioural flexibility and mushroom body volume was examined.

In Chapter 5 I integrate the results and conclusions from the three data chapters with previous research findings on behavioural flexibility and the neural underpinnings of flexibility in bumblebees. I also discuss the applications of my research, both as a starting point for future research, and as information that may aid in bumblebee conservation.

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Chapter 2

2 Development and application of a model of flower handling in bumblebees (*Bombus impatiens*)

2.1 Introduction

All foraging bumblebees are tasked with the identical job of provisioning their colonies with nectar and pollen extracted from flowers, but which flower species they visit varies dramatically across individuals and even across foraging trips (Heinrich, 1976a; 1979a/2004; Kearns & Thomson, 2001). This variation in flower visits across bees is indicative of the generalist foraging strategy typical of most bumblebee species (Kearns & Thomson, 2001). This foraging strategy has the obvious advantages of being flexible and allowing for adaptation to changes in flower availability across geographic locations and seasons, but the advantages of generalist strategies come at a cost (Dall & Cuthill, 1997; Mery & Berns, 2010). The flower types on which bumblebees forage differ greatly in morphology, which in turn creates differences in the location and accessibility of nectar and pollen (Figure 2-1) (Laverty, 1980). Bees must learn how to extract nectar and pollen from each of the different flower species that they visit, referred to as flower handling (Heinrich, 1976a;1976b;1979b; Laverty, 1980). This investment in learning, or gathering information about resources, is the cost of generalism (Dale & Cuthill, 1997; Mery & Berns, 2010).

How is it that bumblebees are able to incur the cost of generalism and learn such a wide variety of handling techniques? Bees have a relatively simple nervous system (Chittka & Niven, 2009), which makes their ability to behave flexibly, a trait often associated with intelligence (Roth & Dicke, 2005), even more remarkable. Although extensively studied from a variety of perspectives, the precise mechanisms of flower handling and the key to bumblebees' flexibility remains undescribed.

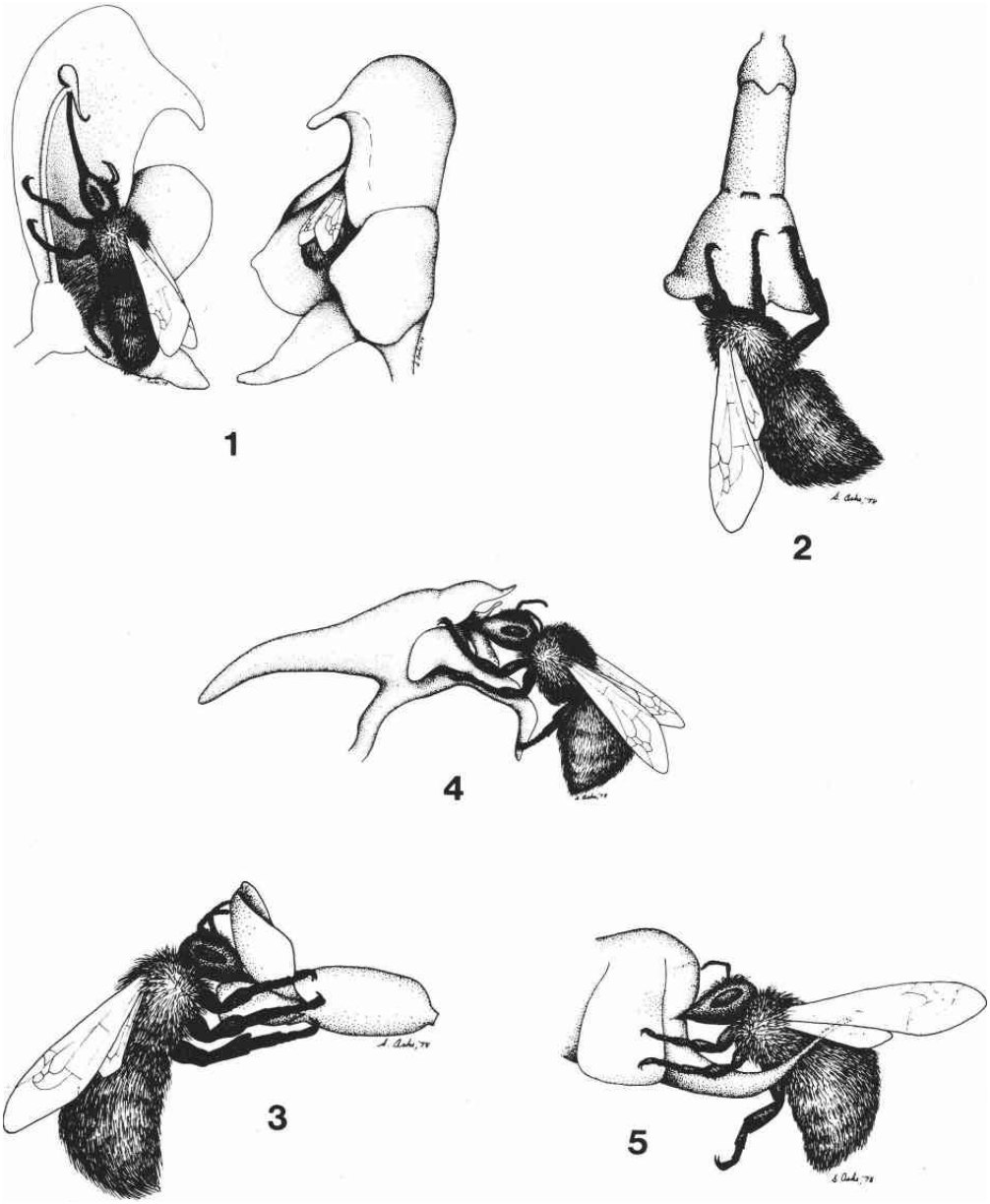

Figure 2-1 Bumblebees depicted foraging on different flower morphologies. The flower species are *Aconitum columbianum* (1), *Mertensia ciliata* (2), *Oxytropis splendens* (3), *Delphinium barbeyi* (4), and *Pedicularis groenlandica* (5). Figure adapted from Laverty (1980).

Flower handling by bumblebees is certainly not a novel research focus, having been identified as a topic of interest by Charles Darwin in his extensive characterizations of the role of pollinators in fertilization of plants (Darwin, 1876). Darwin made two key observations, (1) bees improve their flower handling abilities with experience, and (2) individual insects tend to visit

the same flower species while foraging, which is now referred to as flower constancy. Flower constancy within individuals, and the causes of constancy, has been a dominant theme in work on foraging bumblebees (Free, 1963; Heinrich, 1976b; 1977; Woodward & Laverty, 1992; Gegear & Laverty, 2005; Raine & Chittka, 2007a). However, it is the former of the two, improvement in flower handling with experience, that is of interest in this chapter.

2.1.1 Learning on flower handling tasks

Early investigations of flower handling were done with varying degrees of formality as part of general observations of foraging and pollination on flowers in the wild (Darwin, 1876; Macior, 1966; Weaver, 1965). Despite the lack of direct experimental study, these early works recognized the role of learning in flower handling and consistently advocated for a combined innate and learned mechanism for flower handling (Darwin, 1876; Weaver, 1965; Macior, 1966), a hypothesis that was subsequently supported by experimental investigations that observed the entire course of learning in foraging bumblebees (Heinrich, 1979b; Laverty, 1980). Few of these sources fully describe what is meant by innate, but here we will consider behaviours that arise without learning and are not modified through learning to be innate (Tierney, 1986).

Heinrich (1979b) observed that naïve bumblebees (*B. vagans*) made numerous errors while attempting to extract nectar from flowers, but with experience reached asymptotic performance at 90% accuracy, with accuracy defined as the absence of behaviours that did not result in nectar access. Similar observations were made by Laverty (1980) in three difference bumblebee species (*Bombus flavifrons, B. kirbyellus, B. sylvicola)*. Inexperienced bees made errors in the locations that they looked for nectar, as well as in the behaviours that they engaged in to access flower resources, however, with experience the behaviours of the inexperienced bees converged with that of experienced bees observed foraging in the wild (Laverty, 1980). Laverty (1980)

quantified foraging efficiency not only with behavioural errors, but also with time taken to extract nectar. This additional measure also showed improvement with experience.

The pattern of improvement in flower handling was not consistent across all flower species. Bees made very few errors initially on simple flowers and showed a prolonged period of learning on more complex flowers (Heinrich, 1979b; Laverty, 1980). It was subsequently shown that flower complexity had a significant influence on acquisition of flower handling (Laverty 1994). Flower species can be placed into three broad types of complexity, (1) exposed nectaries, (2) nectaries at the base of a long corolla tube, and (3) nectaries that are blocked by overlapping petals or unusually placed (Laverty, 1994). Bees take the longest time to acquire competency on flowers with closed entrances that required bees to push apart petals to access nectaries (Laverty, 1994).

Another noteworthy observation in these early investigations of flower handling was that the errors observed in naïve bumblebees were not random (Laverty, 1980; Laverty, 1994). Bees' tended to direct their erroneous behaviours at particular parts of floral inflorescences, such as points of petal convergence (Laverty, 1980). This pattern of non-random errors has not been consistently observed, with Weaver (1956) observing that bees foraging on novel flowers appeared to probe areas at random, however, the implication that there is an innate preference to direct behaviours to particular regions of flowers is important in consideration of the mechanisms of flower handling. Additionally, analysis of naïve bumblebee flower approach behaviours in the presence of a various visual and olfactory cues has shown that some landing and probing responses are guided by innate responses to flower properties (Lunau, 1991). These findings support the uncontroversial view that innate processes are important in flower handling and it is not entirely a trial and error driven learning process wherein a foraging bee would be

akin to one of Thorndike's cats (1911/1970), attempting to get into the puzzle box rather than out.

The numerous demonstrations of improvement in flower handling with experience in bumblebees (Heinrich, 1976b; 1979b; Laverty, 1980; 1994) and the clear role of innate processes in foraging make it almost irrefutable that flower handling results from a combination of innate predispositions and learning. However, there remains considerable ambiguity about the content of both the innate or learned component. Are the innate processes limited to the approach behaviours identified by Lunau (1991) or do they extend to the actual manipulation of the flower? Does the learned component consist of novel behaviours or just a modification of existing innate behaviours? Laverty (1994) suggested that the difference in performance on simple and complex flowers was a result of programed responses being sufficient to extract nectar from simple flowers, but that programed responses were not sufficient to access rewards in complex flowers and additional behaviours had to be learned. This is a more fleshed-out theory, but it still falls short of a full characterization of flower handling learning.

2.1.2 Modeling motor learning in the lab

There are a number of investigations of motor skills in bumblebees that involve observing behaviour on artificial foraging tasks that sometimes bear very little resemblance to handling real flowers (Chittka & Thomson, 1997; Chittka, 1998; Mirwan & Kevan, 2014; Alem et al., 2016). Some of these tasks are explicit attempts to model flower handling and natural foraging behaviours (Chittka & Thomson, 1997; Chittka, 1998) and others are specific attempts to test bumblebees on tasks dissimilar to any they might naturally encounter (Mirwan & Kevan, 2014; Alem et al., 2016). Given bees' flexibility when initially foraging (Heinrich, 1976a; 1979a/2004), the large morphological flower variation that they are able to handle (Heinrich,

1976a; Laverty,1980), and their ability to forage on non-native flowers that they could not possibly have encountered in their previous foraging experience (Heinrich, 1979b), it is likely that all of the tasks, regardless of the intent of the researchers, are measuring flexible use of natural foraging behaviours. The learning and motor system involvement in all of these investigations makes them applicable to understanding flower handling.

Chittka & Thomson (1997) developed an apparatus in which bumblebees needed to turn either left or right in a t-maze with the correct direction indicated by a colour stimulus at the apparatus entrance. The task was akin to bumblebees entering a flower and then reorienting within the corolla to access nectar rewards. Similar to observations of bees when foraging on natural flowers, error rates of naïve bees in early trials were high and handling times were long. With experience bees did improve to asymptotic error rates that were comparably low as those observed in more natural foraging conditions, around 5%. Bumblebees completed hundreds of trials on the task to reach asymptotic performance, but when looking exclusively at directional errors (i.e. instances when bees turned the wrong way) significant improvement on the task occurred in the first 10 trials. This rapid improvement observed under controlled laboratory conditions is an indication that although previous observations had included large numbers of trials it may only be necessary to observe early trials to characterize learning on flower handling tasks.

In addition to the numerous attempts to study flower handling in the laboratory there has recently been a good deal of research on non-natural complex motor learning in bumblebees (Mirwan & Kevan, 2014; Alem et al., 2016). This work is largely an attempt to extend the study of physical cognition, popular in primates (Povinelli, 2003; Emery & Clayton, 2009) and some avian species (Seed et al., 2006; Emery & Clayton, 2009), to bumblebees. The tasks used to look

at physical cognition in bumblebees range from pushing aside a centrifuge tube lid (Mirwan & Kevan, 2014) or ball (Mirwan & Kevan, 2014), to pulling on a string (Alem et al., 2016), and demonstrate large flexibility in bumblebee motor learning. However, bees in these studies were trained in a step-wise fashion to perform the required motor behaviours, which limits the applicability of the findings to studies of flower handling. When extracting nectar and pollen from flowers foraging bumblebees must successfully manipulate the entire flower, without the benefit of rewards for intermediate accomplishments. Despite the limited information about bumblebees' natural foraging behaviours that can be learned from training them on non-natural tasks, they do provide information on the range of motor behaviours that bumblebees will exhibit in pursuit of rewards and are therefore helpful in the development of any laboratory models of motor behaviour in bees.

2.1.3 Current study

Investigations into how bumblebees are able to forage on a wide variety of flower morphologies, demonstrating both flexibility and expertise, have been extensive, but nonetheless insufficient to fully characterize flower handling learning. The assumption of researchers has been that flower handling has both innate and learned components, but understanding of the contents of either of these processes is incomplete. The goal of the current study was to develop a model of flower handling behaviour in the laboratory and measure the performance of bumblebees on the task in a way that allowed for the quantification of both innate and learned components of flower handling.

2.2 Experiment 1 – Model Development

The first experiment in this chapter develops the flower handling model. The goals of this experiment were to confirm that the apparatus could be successfully solved by bumblebees, and

to provide an initial characterization of bee behavior on this complex flower handling task. The apparatus was intended to model the most complex flower type described by Laverty (1994), wherein the bee must move petals to access the nectary. Given that experienced wild bees converge on one solution when foraging on these complex flowers (Laverty,1994) the apparatus was designed to have a single solution and accurately model natural complex flowers. The apparatus was also designed to be solvable by bees without shaping given that bees foraging in the wild are not reinforced for incomplete components of handling on complex flowers.

2.2.1 Methods

2.2.1.1 Subjects and housing

Subjects were bumblebee foragers (*Bombus impatiens*) from 3 colonies acquired from Biobest Canada Ltd. (Leamington, ON). Bees were housed in a 3.0 X 8.5 m room within 24h of arrival at Western University (Figure 2-2). Biobest colony boxes consisted of a plastic box within a cardboard box (24(w) X 30.5(l) X 20(h) cm) and were placed on a table (0.9 X 1.8 m) in the housing room and the built-in entrance/exit door in the colony box was opened to allow bees to exit the colony and forage without restriction in the room. The bees were provided with *ad libitum* pollen directly in the colony, and *ad libitum* 20% sucrose solution available from five foraging patches within the room. Each foraging patch consisted of a white 30.5 X 30.5 cm Smoothfoam™ polystyrene sheet and five artificial flowers. The artificial flowers were made from clear 7ml plastic microtubes (Axygen®,Union City, CA) with a clear plastic corolla approximately 5 cm wide.

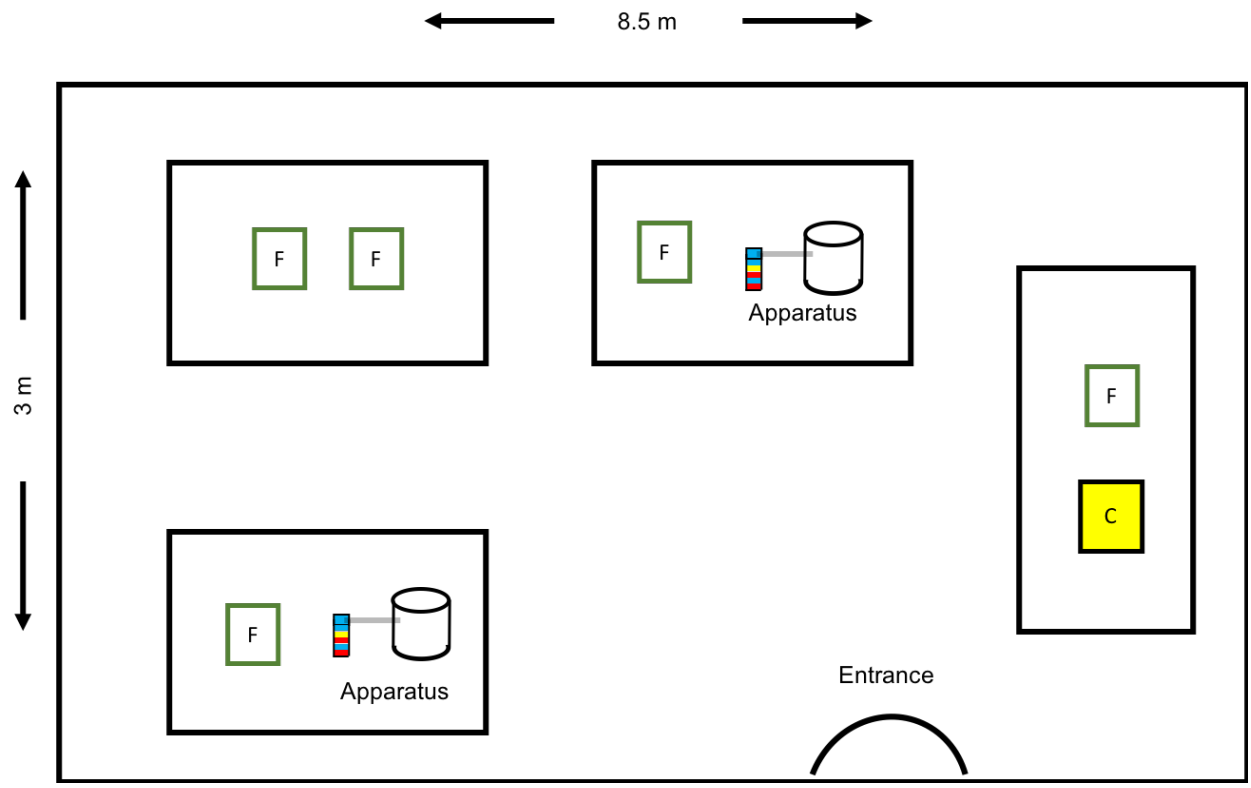

Figure 2-2 Bumblebee housing room. The bumblebee colony box (C) was placed on a table within the housing room and bumblebees were given unrestricted access to the room. Each artificial foraging patch (F) contained five artificial flowers supplied with 20% sucrose. The two testing apparatuses are shown in their positions in the room. The apparatuses remained in the housing room throughout the entire experiment.

Individually bees were tagged for identification with either plastic number tags (Betterbee Inc., Greenwich, NY) or with Posca paint markers (Mitsubishi Pencil Co.). The tagging procedure involved collecting bees in a specially designed tagging apparatus that immobilized bees between a sponge and plastic mesh allowing the tag to be applied to the bee. All bees were tagged while engaging in foraging trips.

2.2.1.2 Apparatus

There were two testing apparatuses available to bees in the housing room. Each apparatus was on a separate 0.9 X 1.8 m table with a foraging patch in front that encouraged bees to visit

the area of apparatus. The two apparatuses were identical and differed only in location in the room.

The apparatus (Figure 2-3) consisted of a 2 cm diameter Perspex® tube with two slots in it in which 'doors' fit smoothly. The tube was elevated between a stack of LEGO® blocks and a circular 13.2 litre white tub (28 cm diameter) manufactured by M & M Industries Inc. The tub had a hole through one side in which the tube fit tightly, which served to immobilize the tube during testing. The end of the tube that was elevated by LEGO® blocks was not immobilized, but did not move during testing due to its attachment to the tub at the other end. There were artificial flowers at both the entrance to the tube and the exit into the white tub which could be baited with sucrose solution. The doors that were used consisted of white construction paper pieces cut in a rectangular shape with a semi-circle end that fit the interior shape of the tube. The doors fit through the slots in the tube, and made contact with the bottom of the tube, but did not conform perfectly to the bottom of the tube. This imperfect fit was essential to the task as it allowed bees to sit their legs in between the door and the tube.

2.2.1.3 Pre-training

The colony was placed in the housing room and given 1-2 weeks to transition to foraging on the sucrose available in the testing room. Once the colony was sustaining itself through foraging on the sucrose provided, tagging and testing began.

Individual bees were habituated to the testing chamber by foraging freely through the apparatus when the apparatus was not in use. The apparatus was baited in two locations, at the entrance to the tube and at the exit, where the reward would be provided during testing. Only bees that had been observed making habituation flights through the apparatus were tested.

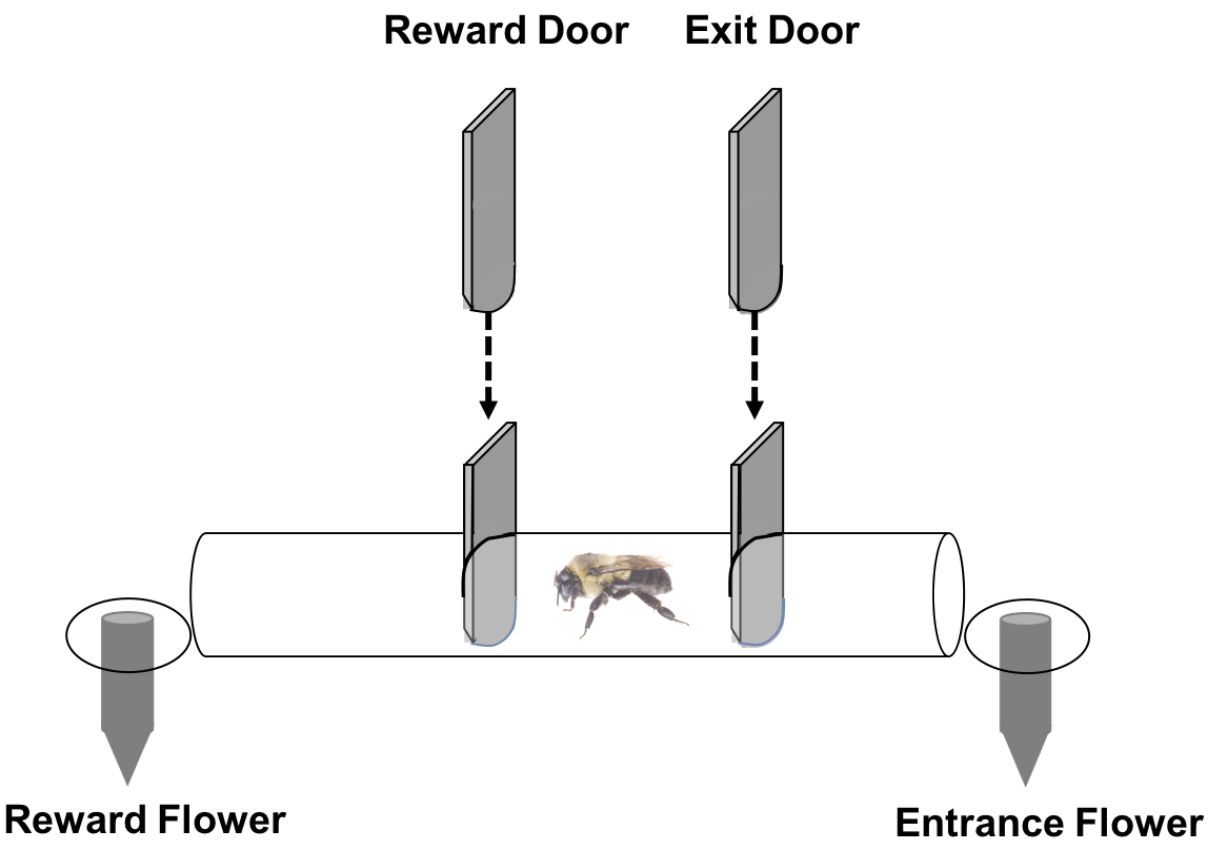

Figure 2-3 Apparatus used in Experiments 1 and 2. The apparatus consisted of a 2 cm diameter Perspex® tube with two slots in which 'doors' fit smoothly. Artificial flowers at the entrance and exit were baited as needed. In Experiment 1 both of the doors were white construction paper. In Experiment 2 the reward door was white construction paper and the exit door was a metal door that could not be opened by the bees.

2.2.1.4 Testing Procedure

At the start of testing sessions free foraging bees were observed and bees that were making trips to the apparatus were identified as candidates for testing and tagged. Once a bee was identified for testing the apparatus was cleared of all other bees by both removing the lid on the reward bucket and placing metal un-openable doors in the tube so that bees could not enter. The reward location was then baited with sucrose, and the sucrose was removed from the entrance.

When a tagged bee approached the apparatus experimenters removed the metal doors from the tube to allow the bee to enter. Once the bee was in the tube two construction paper doors were inserted into the tube, one that could be opened to access food reward and one that could be opened to exit. Trials ended either when the bee successfully opened one of the doors, or when 300 seconds had passed. A video was taken of each trial using a HERO3 video camera (GoPro Inc., USA). Following opening a door the bee was allowed to either leave the apparatus, if the exit door was opened, or to fill to repletion on the reward flower if the reward door was opened. Once the bee had completed a trial and left the apparatus the apparatus was reset for the next visit by a tagged bee.

Bee identification, time of day, and trial duration were all recorded by hand during testing and all other measures were collected during video analysis.

2.2.1.5 Video scoring

Videos were scored using Observer XT, which allowed the total duration of each motor behaviour within a trial to be quantified. Latency to success was measured from the video recordings in order to get a more accurate measure than those taken during testing sessions. Latencies for each trial began when bees entered the apparatus and the door was inserted to close them into the apparatus. It was found that bees could open the apparatus door by either lifting the door and sliding upside down underneath, or by lifting the door entirely out of the apparatus. In order to obtain a latency score that was accurate across these solution types, latency was stopped when bees had 50% of their bodies under the door, a position which always resulted in successfully opening the door. On trials where bees failed to successfully open a door their latency was recorded as the max trial time (300s). Four distinct behaviours, which will be identified and described in the results section, were observed on the video recordings. The

durations of two of the four behaviours on each trial were quantified. The two behaviours were scored as mutually exclusive. The measurements taken during video scoring were used to calculate the proportion of time spent inverted and the proportion of time spent pushing for each trial for each bee.

2.2.1.6 Data Analysis

In order to account for large individual differences in performance, difference scores were calculated for each bee for every trial, where their performance on the first trial was subtracted from their performance on each subsequent trial. Individual differences were large across all three measures of performance (i.e. latency, time inverted, and time pushing), so difference scores were calculated for each. The difference scores were used for both figures and analyses.

All data were analyzed using IBM® SPSS®. When ANOVA was used Greenhouse-Geisser corrected values were used if sphericity assumptions were violated.

2.2.2 Results

A total of 42 bees completed trials in the apparatus and of those, 33 completed at least one successful trial in which they lifted a door to either leave the apparatus or obtain a reward. Statistical analysis is restricted to 12 bees that each completed multiple trials. The first 9 trials completed by each bee were used in the analysis, because too few bees completed more than 9 trials to analyze. Of the trials included in the analyses, there were 13 trials in which the bee failed to open one of the doors. The inter-trial-intervals were highly variable across and within bees ranging from 4min to 24h.

2.2.2.1 First trial performance

Videos of the first trial by all 12 bees were viewed to establish a behavioural repertoire for *Bombus impatiens* on the task. Four behaviours were identified as part of the bumblebees' behavioural repertoire; (1) inverting, defined as the bee having greater than 50% of its underside exposed, (2) pushing, defined as appearing to apply pressure when one or more legs are touching the door while right side up, (3) proboscis extension, defined as clear extension of the proboscis, and (4) biting, defined as biting the door with the mandibles. Bees were initially biased towards either inverting, pushing, or both as bees were more likely to engage in these behaviours in the first 30s of the trial than either proboscis extension or biting ($X^2(3) = 9$, $p = .029$) (Figure 2-4). It was confirmed that inverting was the only behaviour that would result in successfully opening the door. This strategy was used in all successful trials.

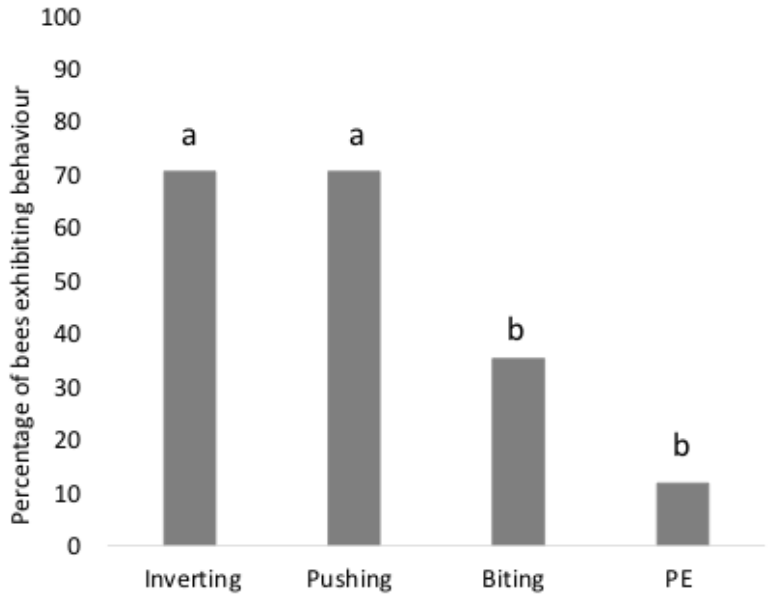

Figure 2-4 Percentage of bees showing each behaviour in the first 30s of the first trial. Significantly more bees spent time flipped and pushing in the tube when compared to biting or proboscis extension (PE). Bars with different letters differ significantly.

2.2.2.2 Performance across trials

Based on the behavioural repertoire established in analysis of the first trial performance it was decided that latency to success, time inverted, and time pushing would be scored for all trials as a measure of learning and change in behaviour across trials. The scale of movement involved in proboscis extension and biting meant that it was not possible to accurately score these behaviours with more detail than presence or absence.

2.2.2.2.1 Latency

Repeated measures ANOVA was used to analyze change in latency across trials. There was no change in performance across trials ($F(8,88) = .93$, p=.49)(Figure 2-5).

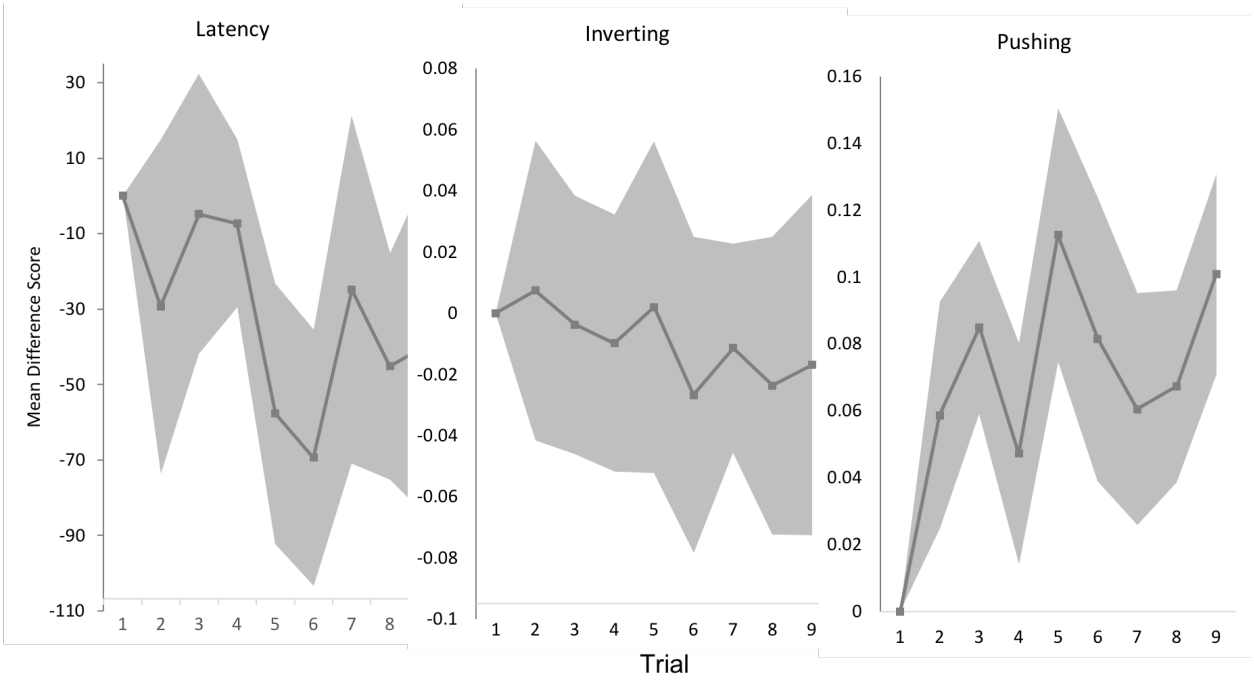

Figure 2-5 Latency, time inverted, and time pushing for Experiment 1. Mean difference scores are indicated by lines and standard error of the mean is shown with shading. Changes in latency, proportion of time inverted (Inverting), and proportion of time pushing (Pushing) were all non-significant.

2.2.2.2.2 Time inverted

A repeated measures ANOVA showed no change in performance across trials ($F(8,88)$ = 0.148, p = .99)(Figure 2-5).

2.2.2.2.3 Time pushing

A repeated measures ANOVA showed that time pushing did not change across trials ($F(8,88)$ = 1.691, p = .11)(Figure 2-5).

2.2.2.2.4 Individual differences

Analysis at the level of individual bees revealed that three bees showed reductions in latency across trials that were significant in logarithmic regression analysis (Bee 1: R^2 = .807, $F(1,7)$ = 29.2, p < .001; Bee 2: R^2 = .711, $F(1,7)$=17.2, p = .004; Bee 3: R^2 = .664, $F(1,7)$ = 13.8, p = .007) (Figure 2-6).

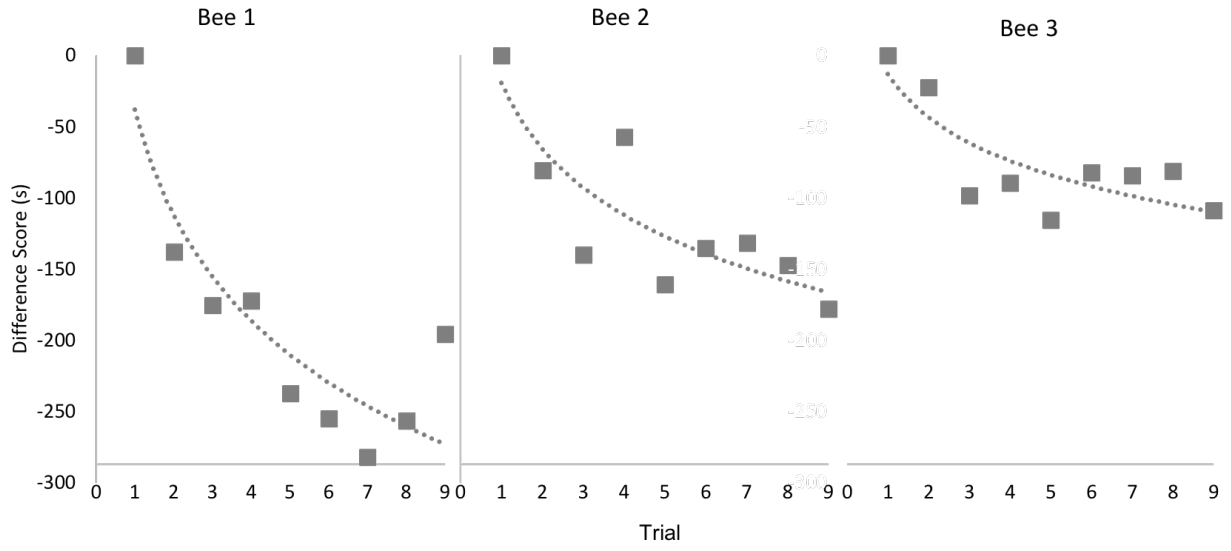

Figure 2-6 Individual latency difference scores for three bees from Experiment 1. Latencies from each of the three bees depicted show significant logarithmic regressions across trials.

2.2.2.3 Bee size and latency

In order to test for an influence of body size on performance head cap width was used as a measure of body size (Mares, Ash, & Gronenberg, 2005) and compared to performance on trial 1. Linear regression revealed no relation between head size and first trial performance (R^2 = .065, $F(1,11)$= 0.696, p = .42).

2.2.3 Discussion

The primary goal of this experiment was to develop a model of flower handling in the lab that could be used for subsequent exploration of flower handling. Multiple bees were able to successfully open the door in the apparatus, willing to complete multiple trials, and converged on the same solution. Additionally, a subset of bees showed learning trends, as measured by reduced latency, across trials. These findings show that development of the model was successful.

The same four behaviours (inverting, pushing, proboscis extension, and biting) were generated by all bees when the door was encountered in the apparatus. The universal nature of these behaviours strongly suggests that they are part of an innate repertoire of behaviours. The presence of an innate component to flower handling has been widely proposed (Darwin, 1876; Weaver, 1965; Macior, 1966; Heinrich, 1979b; Laverty, 1980), but there has been little consideration of exactly what that innate component might be. These data are consistent with the innate component being a set of motor patterns initiated when encountering a flower petal while trying to access nectar.

Improvement with experience has been a very consistent finding of flower handling research (Heinrich, 1976b; 1979b; Laverty, 1980, 1994), however there was no overall effect of experience found here. In previous observations of flower handling, bees made multiple flower visits in a single foraging trip and multiple foraging trips in quick succession (Heinrich, 1976b; 1979b; Laverty, 1980, 1994). In this experiment bees were tested opportunistically, making only one flower visit in each foraging trip and experiencing variable delays of up to multiple days. Therefore, it is possible that the highly variable testing schedule had an influence on performance and interfered with bees' ability to improve across trials.

Although there was no overall effect of experience on performance, there were three individual bees that did show significant improvement across trials. This finding makes it likely that learning, as measured by improvement in performance, is possible on the task and that variation in testing conditions across bees may have affected the results.

Preliminary use of the model in this experiment was successful, in that bees were successful in opening the apparatus. However, the opportunistic self-initiated testing schedule for bees and consequently highly variable inter-trial intervals (ITIs) interfered with my ability to draw clear conclusions about the involvement of learning in bees' performance.

2.3 Experiment 2 – Flower Handling Learning & Forgetting

Experiment 2 was designed to resolve the problems with highly variable ITIs that occurred in Experiment 1. The efforts to increase control over ITIs included a change in housing that reduced physical distance between the colony and the apparatus as well as changing testing procedures to eliminate the disruption of testing multiple bees at the same time. In this experiment bees were tested one at a time for a specific number of trials over the course of multiple days. The goal of this experiment was to test bees in a standardized way that allowed for complete characterization of the acquisition and forgetting of a motor behaviour similar to complex flower handling. Although trials were still self-initiated and variable ITIs could not be controlled entirely, the change in testing procedure dramatically increased the consistency in testing schedules.

2.3.1 Method

2.3.1.1 Subjects & housing

Subjects were 15 *Bombus impatiens* foragers from four Biobest colonies.

Colony boxes were attached to 122 X 101.5 X 66 cm foraging chambers within 24h of arrival at Western University. The foraging chambers consisted of a wooden frame with wire mesh sides and a wooden floor on which foraging patches were located. Bees had 24h access to the foraging chamber through a Perspex tube that joined the colony box to the foraging chamber. The bees were provided with *ad libitum* pollen directly in the colony, and ad libitum 20% sucrose solution available from four foraging patches within the foraging chamber. The foraging patches were identical to those used in Experiment 1.

As in Experiment 1, bees were tagged using coloured number tags (Betterbee Inc., Greenwich, NY) and Posca paint markers (Mitsubishi Pencil Co.). Only bees that entered the testing apparatus were tagged.

2.3.1.2 Apparatus

There were two testing apparatuses available to bees. The apparatuses were on an adjoining table to the foraging chamber and attached directly to the foraging chamber. The two apparatuses were identical and differed only in location.

The Perspex tube component of the apparatuses were identical to those used in Experiment 1 (Figure 2-3). The tubes were attached to the foraging chamber at their entrance and to a small plastic reward box at their exit. The boxes measured approximately 5 cm^3 and were constructed from beige corrugated plastic with a clear plastic lid. The boxes contained two lids removed from clear 7ml plastic microtubes (Axygen®,Union City, CA) that were upturned to hold a sucrose reward. The lids were used instead of the full microtubes that acted as artificial flowers in Experiment 1 because of the limited space in the reward boxes. Bees were able to enter the reward box, collect sucrose, and then exit the apparatus through the Perspex tube.

Doors used in this experiment were constructed from white plastic coffee cup lids (SOLO®) or metal and shaped identically to those used in Experiment 1.

2.3.1.3 Pre-training

During a pre-training phase all foraging bees were given access to the apparatuses. The apparatuses were baited at the entrance and the exit with high valued sucrose (40%). The pre-training phase lasted a minimum of 1 week and continued throughout testing when a testing session was not in progress.

2.3.1.4 Testing procedure

At the start of a testing session the apparatuses were baited with high valued sucrose (40%) and the bees were observed foraging. The experimenter then identified a tagged bee making regular foraging trips and began the testing session. During testing sessions only the bee that was currently being tested was given access to the apparatus. Other bees were prevented from entering by inserting metal doors.

At the start of each trial the plastic reward door was inserted into the tube and the metal exit door was removed to allow the bee access to the apparatus. Once the bee entered the apparatus the metal exit door was inserted into the tube to prevent the bee from exiting the apparatus. Only the plastic door could be opened by the bee, which meant that bees had to encounter the reward box, and a sucrose reward, before the experimenter removed the metal door and allowed them to exit. This change in procedure was made to ensure that each successful opening of a door resulted in access to sucrose reward. Trials had a maximum time of 300s, after which the plastic door would be removed by the experimenter providing access to the sucrose reward. The plastic door was never removed when the bee was in contact with the door to avoid reinforcing unsuccessful behaviours. Once bees had filled to repletion on sucrose in the reward

box, either following a successful or unsuccessful trial, the metal door was removed and they were allowed to exit the apparatus.

Testing sessions consisted of 10 trials. Bees that continued to forage on the days following their initial testing session were tested for three sessions over three days. Each session consisted of 10 trials.

As in Experiment 1, video recordings were taken for all trials, with bee identity, time of day, colony, and preliminary latencies recorded by hand during testing.

2.3.1.5 Video Scoring

As in Experiment 1, videos were scored using Observer XT. Scoring was adjusted to reflect the change in testing procedure wherein only one door was now openable. Videos were scored for latency to success, time inverted, time pushing, and time spent attempting to exit. Latency was scored identically to Experiment 1. Time inverted was defined as time with more than 50% of the bee's underside exposed while in contact with the plastic door. Time pushing was defined as applying pressure when one or more legs are touching the plastic door while right side up. Attempting to exit was defined as any time spent in contact with the metal exit door.

2.3.1.6 Data Analysis

Repeated measures ANOVA was conducted in IBM® SPSS® to individually analyze latency, proportion of time inverted, proportion of time pushing, and time interacting with the exit, for both the first session and the data across all three sessions. Analysis of the relation between the different dependent measures was done using repeated measures correlation analysis in RStudio®.

2.3.2 Results

Fifteen bees completed a session of 10 trials, and of those bees 10 completed additional testing sessions of 10 trials on each of the two subsequent days. Analysis of the first 10 trial session and analysis of trends over the course of the three testing sessions were done separately.

Similar to Experiment 1, large individual differences occurred in this experiment. Consequently, scores were analyzed as difference scores calculated in relation to each bee's initial performance.

The ITIs within sessions ranged from approximately 2min to 20min.

2.3.2.1 Performance in the first session

2.3.2.1.1 Latency

There was a significant reduction in latency across trials ($F(3.59, 50.2) = 13.7$, $p < .001$)(Figure 2-7).

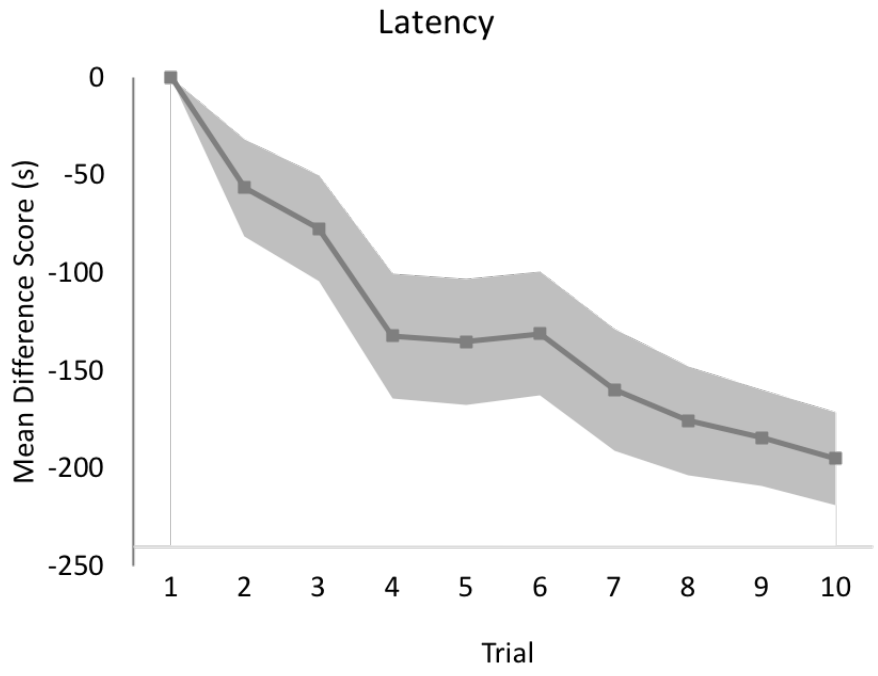

Figure 2-7 Latency difference scores for trials 1-10 in Experiment 2. Mean latency difference scores for bees are represented by the line and the shading indicates standard error of the mean. There is a significant decrease in latency across trials.

2.3.2.1.2 Time inverted

Time inverted significant increased across trials ($F(9, 68.1) = 3.58$, $p = .007$)(Figure 2-8).

2.3.2.1.3 Time pushing

Analysis showed that time pushing did not change across trials ($F(9,126) = 1.27$, $p = .259$)(Figure 2-8).

2.3.2.1.4 Exit

Time interacting with the exit did not change across trials ($F(4,55.9) = 2.2$, $p = .08$)(Figure 2-8).

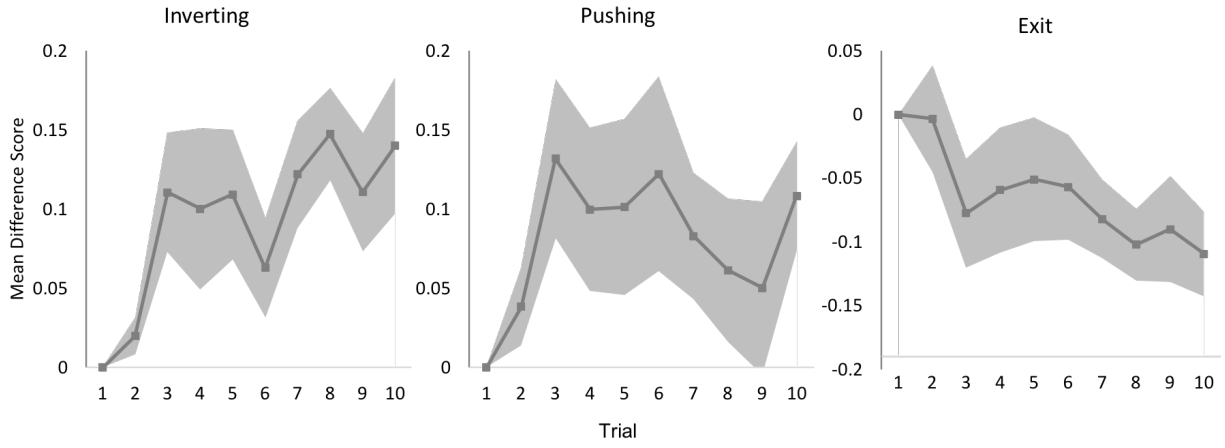

Figure 2-8 Time inverted, time pushing, and time interacting with the exit for trials 1-10 in Experiment 2. The mean difference scores for each behaviour are an average of the difference scores calculated using the proportion of time spent engaging in each behavior by individual bees. Bees significantly increased the proportion of time inverted across trials 1-10, but did not significantly change the proportion of time pushing or attempting to exit. Standard error of the mean is shown with shading.

2.3.2.2 Performance across three days.

2.3.2.2.1 Latency

Latency data was analyzed using repeated measures ANOVA with two within-subjects factors, day and trial. There was a significant main effect of trial ($F(2.82, 22.6) = 3.86$, $p = .025$)

and a significant main effect of day ($F(1.23, 9.8) = 6.78$, $p = .023$) (Figure 2-9). The trial X day interaction did not reach significance ($F(4.12,33) = 2.63$, $p = .051$).

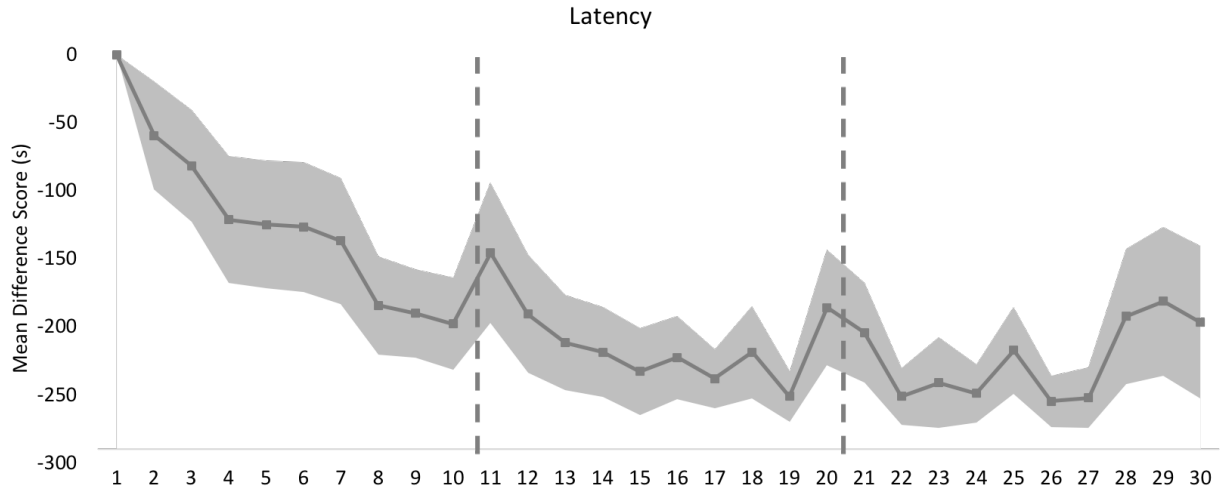

Figure 2-9 Latency difference scores for trials 1-30 in Experiment 2. Vertical dashed lines indicate overnight intersession intervals. There was a significant decrease in latency across trials within each day, and a significant decrease across days. The interaction between trial and day was non-significant. Standard error of the mean is shown with shading.

2.3.2.2.2 Time inverted

Change in time inverted across trials was analyzed using repeated measures ANOVA with day and trial included as within subjects factors (Figure 2-10). There was a significant increase in time inverted across trials ($F(9,72) = 2.57$, $p = .013$) and a significant increase across days ($F(2, 16) = 5.89$, $p = .012$). The trial X day interaction was not significant ($F(18,144) = .568$, $p = .918$).

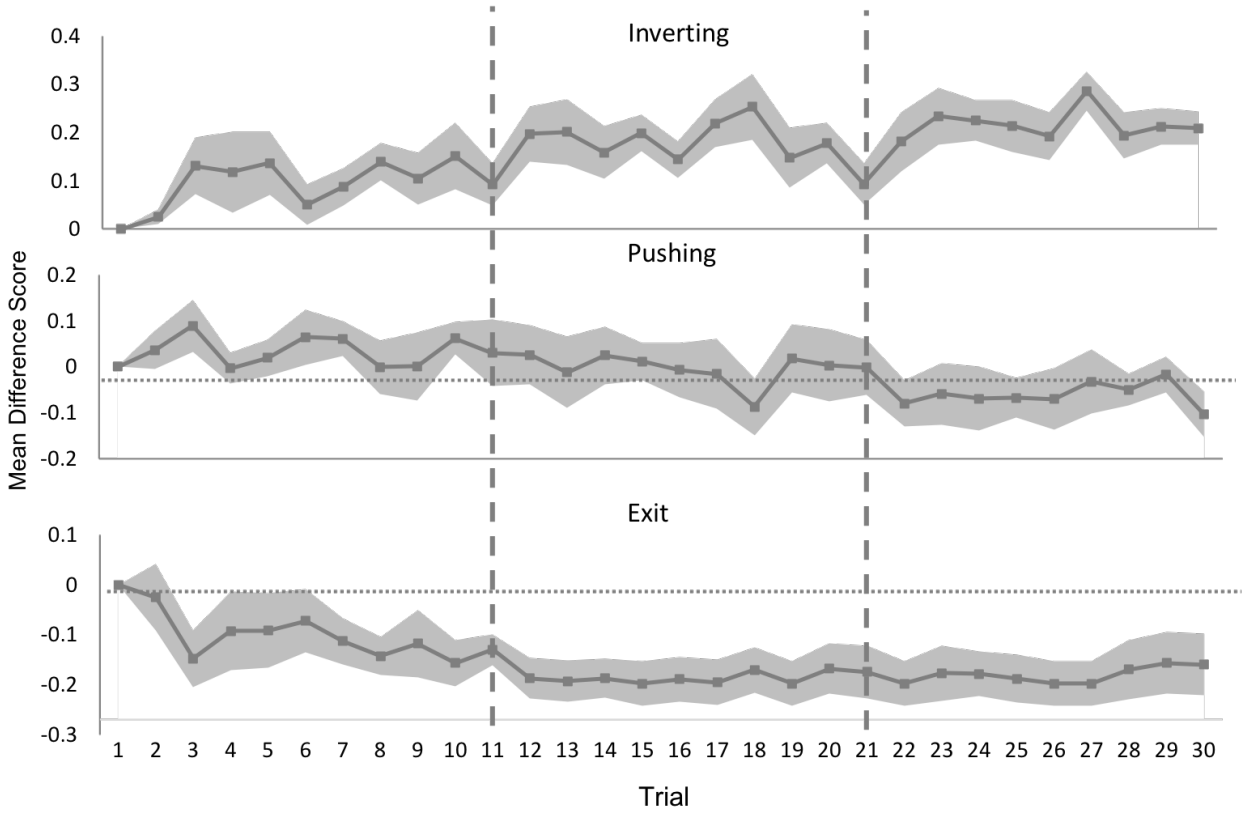

Figure 2-10 Time inverted, time pushing, and time interacting with the exit for trials 1-30 in Experiment 2. Vertical dashed lines indicate overnight intersession intervals. Horizontal dotted lines in the Pushing and Exit panels indicate performance on trial 1, the comparison point for the difference scores. In the Inverting panel initial performance is represented by the horizontal axis. Bees showed a significant increase in proportion of time inverted both across trials and across days. The interaction between trial and day was not significant. There was a significant effect of day on time spent pushing, but no significant effect of trial or the trial X day interaction. There was a significant effect of day for time spent at the exit, but significant effects were not found for trial or the trial X day interaction. The mean difference scores for each behaviour are an average of the difference scores calculated using the proportion of time spent engaging in each behavior by individual bees. Standard error of the mean is shown with shading.

2.3.2.2.3 Time pushing

Time pushing was analyzed using repeated measures ANOVA with day and trial included as within subjects factors (Figure 2-10). There was a significant main effect of day ($F(2,16)$ = 4.88, p = .022), but neither the main effect of trial ($F(9, 72)$ = .687, p = .719), nor the trial X day interaction were significant ($F(18,144)$ = .748, p = .756).

2.3.2.2.4 Exit

Change in time spent interacting with the exit across trials was analyzed using repeated measures ANOVA with day and trial included as within subjects factors (Figure 2-10). There was a significant main effect of day ($F(1.2,9.56) = 6.5$, $p = .026$), but neither the main effect of trial ($F(3.47, 27.8) = 1.87$, $p = .150$), or the trial X day interaction were significant ($F(3.59, 28.7) = 1.644$, $p = .195$).

2.3.2.3 Correlations between dependent variables

Relations between latency and proportion of time inverted, as well as latency and proportion of time inverted, were quantified by calculating repeated measures correlations using the R package rmcorr: Repeated Measures Correlation (Bakdash & Marusich, 2017). This analysis was done using the raw data for both latency and proportion of time inverted or pushing rather than the difference scores that were used in previous analysis.

A significant negative correlation was found between latency and proportion of time inverted for both the first session ($r(134) = -.357$, $p < .001$) and across the three sessions ($r(260) = -.48$, $p < .001$) (Figure 2-11). A significant negative correlation was found between latency and proportion of time pushing for the first session ($r(1341) = -.202$, $p = .019$), and a positive correlation was found between latency and proportion of time pushing across the three sessions ($r(260) = .329$, $p < .001$) (Figure 2-11).

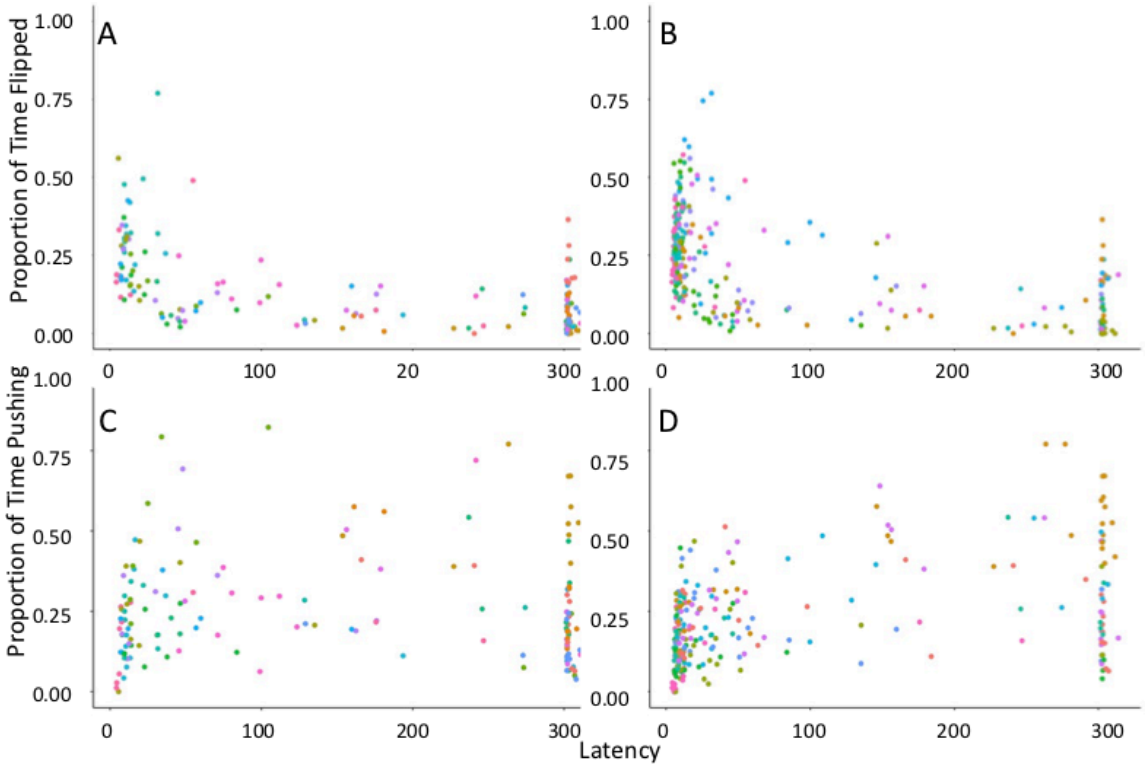

Figure 2-11 Correlations between dependent variables. The relation between latency and proportion of time inverted on the first 10 trial session is shown in panel A. Latency and proportion of time inverted across three 10 trial sessions is shown in panel B. Latency and proportion of time pushing for the first session is represented in panel C, and across the three sessions in panel D. The data from each bee in the analysis is represented by a different colour. All four relations are significant. Latency and proportion of time inverted had a negative correlation in both the first session and across all three sessions. Latency and proportion of time pushing were negatively correlated in the first sessions, but had a positive correlation across all three sessions. Each colour in a panel represents the data from one bee.

2.3.3 Discussion

The goal of Experiment 2 was to expand on the findings in Experiment 1 by testing bees on the model of flower handling under more controlled and consistent conditions, to provide complete characterization of learning trends on the task. Bees completed three sessions across three days, which provided a measure of change over days, in addition to acquisition information across trials.

Bees showed significant learning across trials, as measured by latency to success, in the first session and across the three days. This supports the hypothesis that the failure to find overall significant learning on this task in Experiment 1 was due to inconsistencies across bees in testing schedules. Learning across trials is a highly consistent finding in flower handling research (Heinrich, 1976b; 1979b; Laverty, 1980, 1994), making its observation in this experiment essential for the apparatus to be considered a model for flower handling.

Bees retained learning from the first session overnight and had lower latencies on days two and three. However, the rate at which they learned on the second two days did not differ from the rate in the first session on day one, as shown by the absence of an interaction. Chittka (1998) showed perfect overnight retention in bees on a foraging task that modelled flower handling, so retaining information overnight is consistent with previous observations of motor tasks. The proportion of time inverted also showed a significant retention across days, which supports the proposed relation between increased use of the inverting behavior and decreases in latency.

The hypothesis generated as a result of findings in Experiment 1, that learning on flower handling tasks is driven largely by selecting and engaging in a successful motor pattern, is supported by the correlation between use of the successful strategy and improvement on the task. This relation held for both improvement in the first session and across all three sessions. It was expected that bees would undergo extinction for unsuccessful strategies, however, the relation between the unsuccessful strategy (pushing) and latency was ambiguous, with significant negative and positive correlations depending on the dataset.

The bees showed a reduction in time pushing and trying to exit across days, but not a significant decline either in trials within a session. These results show that bees did reduce their

time spent on unsuccessful behaviours, but the decline in use of unsuccessful strategies was not as clear as the increase in use of the successful strategy. Given that bumblebees are generalists, there could be a benefit to maintaining a low level of use of unsuccessful strategies in case foraging circumstances (e.g. flower availability) change and they become successful strategies. Maintenance of unsuccessful flower handling strategies could be similar to the observations that bumblebees occasionally visit other flower species while being largely constant to a single species (Heinrich, 1979b), in that it is a safeguard against changes in foraging conditions.

2.4 General Discussion

In Experiment 1 a novel apparatus was designed to measure flower handling. The apparatus was designed to generate a pattern of learning comparable to real flowers, that is an improvement with experience and eventual convergence on a single successful strategy/motor pattern. Experiment 2 continued assessment of the apparatus as a model for flower handling and involved extensive quantification of bee behaviour on the task, which was then used to characterize learning of flower handling. Bumblebees all demonstrated the same repertoire of behavior when interacting with the apparatus, improved with experience, converged on a single successful strategy/motor pattern, and analysis showed that learning was related to selection of the successful strategy/motor pattern. These findings support the hypothesis that flower handling improvement is due to changes in the selection of innate motor patterns through associative learning.

2.4.1 Innate and learned processes

It has been long assumed that flower handling involves both innate and learned processes (Darwin, 1876; Weaver, 1965; Macior, 1966; Heinrich, 1979b; Laverty, 1980), but the contents and contributions of those two processes were unclear. Here I have shown that the innate

component is the complex motor pattern required to extract nectar and pollen and the learning component is the relatively simple process of associative learning, wherein a particular motor pattern is reinforced to extract nectar and pollen from a particular flower species is reinforced. The combination of innate motor patterns and learned preferences is consistent with the selection pressure on bumblebees to maintain flexibility in foraging (Heinrich, 1976b) and the additional selection pressure to minimize costly learning investment (Burns, Foucaud, & Mery, 2010).

The ability of the contributions of innate and learned processes described here to account for both the flexibility and constancy found in foraging bumblebees can be shown by applying it to differences in specialist and generalist bumblebee species. Specialist bumblebees are more efficient at foraging on their preferred species than are generalist bumblebees (Laverty & Plowright, 1988). The difference in cognition between the two could be an innate preference in the specialist species of bumblebee for a particular successful motor pattern, which would eliminate the need to learn a preference and increase efficiency. Generalist bees would have to acquire the preference for the successful motor pattern, which would account for the difference in handling acquisition between the two species. This would also be a parsimonious explanation for the difference between the species given that differences in cognition would be quantitative and not qualitative.

2.4.2 Nectar vs. pollen

The model developed here involved exclusively nectar rewards, but foraging bumblebees are required to collect both nectar and pollen rewards. Similarly to nectar, collecting pollen from flowers involves complex motor output and varies depending on flower morphology (Thorpe, 2000), so it is worth considering the applicability of the findings here to pollen foraging.

Pollen foraging seems to be largely similar to nectar foraging in that bees can learn associatively based on pollen rewards (Muth, Papaj, & Leonard, 2016), and bees improve their ability to extract pollen rewards with experience (Raine & Chittka, 2007b). These commonalities between nectar and pollen foraging might have resulted in similarly innate and learned processes. However, bees take longer to reach asymptote performance when foraging for pollen than nectar (Raine & Chittka, 2007b). Additionally, pollen foraging can require highly specialized behaviours such as floral sonication (Thorpe, 2000). It is possible that the increased complexity of motor behaviours required for pollen foraging might increase the selective pressure for learning in generalist species rather than investing in innate motor behaviours that may not be as widely applicable as nectar foraging behaviours. An investigation of pollen foraging of the kind described here for nectar foraging would be useful in determining the similarities and differences between these two often conflated types of foraging.

2.4.3 Motor learning

One consistency within the literature on flower handling is the reference to motor learning or motor skills, without a clear definition of either of these terms. Although a perfect definition of motor skills is elusive, the field of motor learning in humans developed from the early definition provided by Pear (1926) describing motor skill as "an integration of well-adjusted performances, rather than a tying together of habits" (p.480) (Pear, 1926; Adams, 1987). Under this definition learning a novel motor pattern would be motor learning, but preferential use of an innate motor pattern as a result of associative learning would not be motor learning. The results described here are consistent with flower handling not involving motor learning, but rather associative learning with a motor component; the actual motor patterns being innate and the preference for successful patterns being learned associatively.

Does this mean that bumblebees are incapable of true motor learning? Given that the motor system of bees is largely analogous to mammals (Chittka & Niven, 2009) it seems unlikely for such a qualitative difference to exist between the two systems. The motor system in bees is simpler than in vertebrates, with fewer pre-synaptic neurons activating motor neurons and fewer motor neurons innervating individual muscles (Chittka & Niven, 2009), and it is possible that this places limits on the motor learning abilities of bees. However, examinations of the number of neurons required to generate complex motor output and to generate novel complex motor output suggest that motor skill learning would not be impossible for bees (Chittka & Niven, 2009). Bees and other invertebrates might learn novel motor patterns for tasks in which flexible complex motor behaviour is under higher selective pressure than the extraction of nectar from flowers, such as changing yaw torque or thrust during flight in response to feedback (Wolf et al., 1992), or when extracting pollen from flowers (Raine & Chittka, 2007).

2.4.4 Conclusion

The goal of this chapter was to develop a model of flower handling for use with bumblebees in the laboratory, and to use that model to further characterize flower handling learning in bumblebees. A model was successfully developed that shared two key features of real flowers, (1) having a single solution and (2) improvement with experience. The model was then used to examine flower handling learning with greater specificity than had previously been done. The resulting characterization involved an innate motor component and a learned bias in the selection of that innate motor component.

The impressive behavioural flexibility demonstrated by bumblebees during acquisition of flower handling can thus be explained with reference only to simple learning mechanisms and highly efficient use of innate mechanisms. This finding not only extends our understanding of

bumblebee learning and behavior, but serves as an example of simple mechanisms generating seemingly complex behavior.

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Chapter 3

3 Revisiting Darwin's Interference Hypothesis

3.1 Introduction

Pollinators are often observed to show consistencies in their flower selection while foraging, referred to as flower constancy (Waser, 1986; Lewis, 1993). Bumblebees are known to display flower constancy while foraging and preferentially visiting a single or small number of flower species (Heinrich, 1979). The benefits of flower constancy for plants requiring pollination are clear (Waser, 1986), but the benefits to bees and other pollinators of restricting their foraging to few flower species is less clear (Chittka, Thomson, & Waser, 1999). A candidate theory, originally put forward by Darwin (1876), is that bees learn how to efficiently extract nectar and pollen from particular flower morphologies and switching between multiple flower morphologies would reduce efficiency. Darwin's hypothesis is now referred to as the interference hypothesis (Goulson, Stout, & Hawson, 1997; Chittka, Thomson, & Waser, 1999) and it persisted as the most likely candidate for flower constancy for many years (Waser, 1986; Lewis, 1993). However, investigations of flower handling interference have failed to show ecologically significant effects of interference (Laverty, 1994b; Raine & Chittka, 2007). Consequently, considerations of flower constancy have largely moved from interference explanations to alternative hypotheses that place less emphasis on flower handling (Chittka, Thomson, & Waser, 1999). Unfortunately, however, we still do not fully understand why flower handling behaviour, which involves learning and is vital to foraging success, is resistant to Darwin's hypothesized interference effects. In this chapter, I revisit Darwin's interference hypothesis to examine why flower handling interference does not play a significant role in flower selection.

3.1.1 Bumblebee foraging behaviour

Darwin described the behaviour of bumblebees, that he referred to charmingly as humble bees, in his extensive writings on pollination (1876). He observed that bumblebees would visit the same species of flower consistently and pass over other flower species (Darwin, 1876). The flower selection of foraging bumblebees has since been characterized more extensively (Free, 1970; Heinrich, 1976; Heinrich, 1979; Chittka, Gumbert, & Kunze, 1997). Aside from *Bombus consobrinus*, which is a specialist species (Laverty & Plowright, 1988), bumblebees forage on a wide variety of flowers (Heinrich, 1976). Bumblebees will engage in a sampling phase at the outset of their foraging career, and then narrow their foraging to a few species (Free, 1970; Heinrich, 1976; Heinrich, 1979). In contrast to honeybees (Free, 1963), bumblebees do not appear to limit their foraging to a single species (Free, 1970; Heinrich, 1976; Heinrich, 1979; Chittka, Gumbert, & Kunze, 1997). Analysis of pollen loads (Free, 1970), and direct observations (Heinrich, 1976), suggest that bumblebees preferentially forage on the same set of flowers even if they are not exclusively foraging on a single species. This behaviour has been referred to as majoring and minoring, with one flower species being the most preferred species for an individual bee and one or more other species visited regularly, but less frequently than the major (Heinrich, 1979). Bumblebees have been observed foraging successfully on six flower species simultaneously in a foraging trip (Chittka, Gumbert, & Kunze, 1997), which seems to suggest an absence of constancy. However, even in circumstances where bees appear to show considerable flexibility in their flower selection, analysis shows that the likelihood of selecting the same species to visit next when leaving a flower is high (Heinrich, 1979).

Taken as a whole, bumblebee foraging involves a period of flexibility during initial flower sampling and then a subsequent period of inflexibility when bumblebees display flower species preferences and flower constancy.

3.1.2 Interference hypothesis

The interference hypothesis was first proposed by Darwin (1876), but it was initially vaguely characterized without any explicit discussion of mechanisms. He compared the performance of a bumblebee to that of a mechanic making multiple engines who makes all parts of one type for all the engines at the same time (Darwin, 1876). This explanation of constancy can be interpreted as describing interference in learning to handle multiple flowers or in being able to switch between different handling techniques that have already been learned (Waser, 1986). Research on the interference hypothesis has been largely on the latter, examining costs of switching between already learned handling techniques (Lewis, 1986; Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998; Goulson, Stout, & Hawson, 1997, Raine & Chittka, 2007.

An established paradigm for exploring the interference hypothesis (Lewis, 1986; Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998) is to train a flower constant insect to asymptotic handling efficiency on one flower species, then train the insect to handle a second flower species. The final step is to once again allow the bee to forage on the original species and compare their handling efficiency to their asymptotic performance after the first phase of training. The difference in handling efficiency following the return to the original flower species is the interference cost, or cost of switching. This paradigm has been used to examine the interference hypothesis in cabbage butterflies (Lewis, 1986), and bumblebees (Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998).

Initial explorations of the interference hypothesis in cabbage butterflies provided support for Darwin's theory (Lewis, 1986), and served to initiate further investigations in bumblebees. Cabbage butterflies were tested in the paradigm described above, with groups subjected to the interference of learning how to handle a second flower species compared to individuals that were not given interfering experience. The butterflies that experienced interference did show a cost of switching when they were tested again on the flower species they initially learned. This cost was not observed in individuals that had a retention interval between foraging attempts on the initial flower species that was matched for duration with time taken to learn the second flower species, but did not learn to handle a second flower species. The results supported Darwin's interference hypothesis as a mechanism for flower constancy in cabbage butterflies (Lewis, 1986). The cost here was measured as 'discovery time' or the duration from landing on a flower until extracting the reward (Lewis, 1986), but additional investigations of costs of switching in butterflies expanded on this measure to include the cost of flight duration (Goulson, Stout, & Hawson, 1997). If improvements in handling efficiency are responsible for constancy then the benefit of handling only a single species must outweigh the cost of flying longer distances to access preferred flowers compared to selecting flowers based on proximity (Goulson, Stout, & Hawson, 1997). It was found that there was a cost in handling time when butterflies switching between flower species, and that no relation existed between switching and travel time between flowers, suggesting a role for handling efficiency in flower constancy, but not travel duration between flowers (Goulson, Stout, & Hawson, 1997). The foundational work on constancy in cabbage butterflies (Lewis, 1986) and follow up work (Goulson, Stout, & Hawson, 1997) both show support for Darwin's interference hypothesis.

Although the work in cabbage butterflies has been interpreted to support the interference hypothesis, the research has been interpreted differently in bumblebees. Woodward and Laverty (1992) used the same paradigm as Lewis (1986) to test Darwin's hypothesis in *Bombus bimaculatus*. Bees showed costs of switching when returning to an initial learned species and showed a preference for visiting flowers that they had just foraged on. A cost of switching paired with a preference for recently visited flower species supports the interference hypothesis, but the size of the switching costs was considered too small to be ecologically significant (Woodward & Laverty, 1992). Woodward & Laverty (1992) used the efficiency costs that occurred in their experiment to calculate the cost of switching after every flower while foraging on 1000 flowers over 40 minutes. Switching after every flower would result in a considerable 19 minute increase in foraging trip duration. However, if the switching rates are made more moderate and field realistic then the cost is considerably reduced. For example, switching every 20 flowers would result in a 1 minute increase in foraging trip duration and a highly constant forager, switching only after 100 flowers, would experience efficiency costs of around 10 seconds. Consequently, the handling cost of switching flower species at field realistic rates was not large enough to explain constancy (Woodward & Laverty, 1992).

Despite the weak explanatory power of switching costs to account for flower constancy, continued investigations of switching costs were conducted to learn more about factors that influence those costs. The morphological complexity of flowers has a significant effect on the time it takes to acquire handling efficiency (Laverty, 1994a), so it makes sense that it would also influence the interference effects on handling. Laverty (1994b) observed bumblebees (*Bombus fervidus*) foraging in patches that consisted of either morphologically simple or morphologically complex flower species. Bee that were foraging in patches with simple flowers switched between

species without any costs in efficiency of handling error rates. However, bees foraging in patches of complex flowers did show costs of switching, indicating that flower complexity is important for interference costs (Laverty, 1994b). The influence of flower complexity was also found when interference effects were measured with the previously used paradigm for testing interference (Gegear & Laverty, 1995). Bumblebees only showed interference effects when both the initially learned flower species and the interfering species were complex flowers (Gegear & Laverty, 1995).

In addition to observations that bumblebees forage on a major and minor flower species (Heinrich, 1979), it has also been shown that they will simultaneously forage on more than two species (Chittka,Thomson, & Waser, 1999). Gegear and Laverty (1998) expanded the interference effects testing paradigm to include more than just a single interference species to address situations in which bumblebees might be foraging on more than just two flower species. Bumblebees showed an accumulation of interference costs with increasing numbers of interfering flower species, and the effect was amplified when the interfering flowers were complex (Gegear & Laverty, 1998). Although handling costs do not seem to be high enough to explain high rates of constancy, it is possible that they are enough to limit the number of species on which bumblebees simultaneously forage (Gegear & Laverty, 1998). Having said that, observations in the field have shown an absence of handling costs even when bees are foraging on more than two species (Raine & Chittka, 2007).

Although an interference effect has been consistently observed under particular testing conditions (Lewis, 1986; Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998; Goulson, Stout, & Hawson, 1997), but when modeled under field realistic levels of switching those costs do not seem large enough to explain flower constancy (Woodward &

Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998). Consequently, Darwin's interference hypothesis remains open for consideration when explaining bumblebee foraging behaviour, but it has thus far shown limited explanatory power.

3.1.3 Task switching in bumblebees

In addition to studying the flexibility or inflexibility of bumblebee foraging behaviour in the context of constancy and ecologically relevant flower handling tasks, there has also been work done on the topic using standard animal cognition paradigms (Strang & Sherry, 2014; Chittka & Thomson, 1997). Reversal learning and serial reversal learning tasks, in which animals are trained on a discrimination between two stimuli and then required to reverse their pattern of behaviour when the reward contingencies are reversed either once (reversal) or multiple times (serial reversal), are frequently used to explore flexibility in animals (Shettleworth, 1998; Shettleworth, 2009). Bumblebees have been shown to be capable of completing serial reversal tasks with both spatial (Chittka & Thomson, 1997) and visual (Strang & Sherry, 2014) discriminations.

Strang and Sherry (2014) tested bumblebees on a standard visual serial reversal paradigm and found that bees were able to reverse their initial associations and with repeated reversals increased the efficiency at which they reversed in response to changes in reward contingencies. This pattern is typical of many species on serial reversal tasks and indicates behavioural flexibility in learning (Shettleworth, 1998; Shettleworth, 2009). There are a number of mechanisms that can account for flexibility in repeated reversal, but the one that fit most with bumblebees' performance was memory interference. Bumblebees showed a reduction in perseverative errors and an overall increased error rate across reversals. Their reduced fidelity to the rewarded stimulus and more frequent responses to the unrewarded stimulus allowed bees to

reverse associations more quickly when reward contingencies were reversed. This pattern of behaviour fit most closely with memory interference as the likely mechanisms behind bees' flexibility (Strang & Sherry, 2014). Bumblebees' performance on this serial reversal task demonstrates that memory interference can play a role in behavioural flexibility during foraging, though a visual discrimination is considerably removed from the fine motor task of extracting nectar and pollen from real flowers.

A reversal task done with bumblebees that has more overlap with flower handling involved running bumblebees through a spatial matching task in a T-maze (Chittka & Thomson, 1997). The design required bees to acquire a spatial association and then reverse that association by using the colour cue provided at the entrance of the T-maze. Bees were required to switch between the two tasks repeatedly, making this a colour cued serial spatial reversal task. Bees were trained on the task on a variety of schedules that including learning exclusively one colour direction pairing (blue → turn right, yellow → turn left), learning two opposite tasks (blue → left, yellow → right) in blocked trials with the two tasks learned one after the other, and a final group that switched tasks after each trial during training. Bumblebees were able to learn the switching tasks and reverse their direction choice in the T-maze using the colour cue (Chittka & Thomson, 1997). Bees that were trained on blocked trials showed an interference effect, measured in errors, when they were required to switch between the two tasks following training (Chittka & Thomson, 1997), which is akin to the interference effects found in previous switching experiments (Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998). Interestingly, the authors attributed the interference entirely to acquisition of the colour association and not to an aspect of the motor component of navigating through the T-maze because errors that bees made following a switch were entirely due to not entering the arm

indicated by the colour cue and not to a failure to successfully navigate the maze (Chittka & Thomson, 1997). Thus, the interference observed in the T-maze was associative not motor, which could have implications when considering the interference effects in fine motor tasks such as flower handling.

3.1.4 Goal of this chapter

Chapter 1 established the hypothesis that learning in flower handling involves forming associations between innate motor patterns and flowers. In this chapter, I apply the hypothesis developed in Chapter 1, as well as the flower handling model, to explore costs of switching. Darwin's interference hypothesis is insufficient to explaining flower constancy shown by bumblebees (Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998; Chittka, Thomson, & Waser, 1999), but how bumblebees avoid significant handling interference costs is still unclear. There are handling costs when bumblebees engage in switching between flowers or model flowers, but those handling costs are surprisingly small (Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998), or in some instances motor costs are entirely absent (Chittka & Thomson, 1997; Raine & Chittka, 2007). Resolving the mystery of how bumblebees avoid interference costs for the learned behaviour of flower handling in the goal of this chapter. There are two points at which a foraging bumblebee could incur a cost of switching. The first is at the point of learning where acquisition of a handle technique might be slower if the bee had previously learned a different handling technique. Experiment 1 explores interference during acquisition by requiring bees to learn two handling techniques and fully characterizing the acquisition of each. The second point of interference occurs when bees repeatedly switch between two different flower types. Experiment 2 addresses the interference that occurs during repeated switching by requiring bees to alternate repeatedly between two

different handling techniques. It is hypothesized that the mechanism for flower handling described in Chapter 1 that allows bees to be flexible in their initial foraging, a combination of innate and learned processes, is the same mechanism that allows bumblebees to avoid interference costs in of switching in their handling techniques.

3.2 Experiment 1 – Task Switching on a Flower Handling Task

Experiment 1 was intended to measure behaviour of bumblebees on a task that captured their natural foraging behaviour of visiting multiple flower species with distinct morphologies. Previous work on bumblebees' switching between two flower types has focused largely on costs in foraging efficiency and flower visit time, both of which are exclusively measures of latency. The goals of this experiment were to analyze switching costs at the level of behavioural change in addition to latency. The apparatus, previously used in Chapter 2 for the initial characterization of flower handling behaviour, was modified to provide two variations of a foraging task. The two tasks were designed to represent two different flower morphologies. They required different motor behaviours for success, but shared general properties of an object that must be manipulated to access nectar. The first variation of the task required the bee to flip upside down (invert) to remove a door (representing a flower petal) to access a nectar reward. The second variation of the task required bees to depress a door down to access a nectar reward.

3.2.1 Method

3.2.1.1 Subjects and housing

Subjects were 20 bumblebees (*Bombus impatiens*) from 1 colony provided by Biobest Canada Ltd. (Leamington, ON). Bee colony boxes were attached to a 122 X 101.5 X 66 cm foraging chamber. *Ad libitum* pollen was provided directly to the colony box and foragers had 24/7 access to *ad libitum* 20% sucrose solution available from four foraging patches in the

foraging chamber. The foraging patches were white 30.5 X 30.5 cm Smoothfoam™ polystyrene sheets and each contained five artificial flowers made from clear 7ml plastic microtubes (Axygen®,Union City, CA). Each artificial flower had a 5cm wide clear plastic corolla.

Bees were tagged for identification with Posca paint markers (Mitsubishi Pencil Co.). Tagging was done while bees were pre-training. Bees were allowed to enter the apparatus and reach the baited reward chamber. While bees were collecting sucrose solution they were tagged on their thorax with a unique combination of colours.

3.2.1.2 Apparatus

Two identical apparatuses were attached to the foraging chamber for pre-training, but only the apparatus farthest from the colony entrance was used during testing. The apparatus was a modified version of the flower handling task developed in Chapter 2 (Figure 3-1) and consisted of a 2 cm diameter Perspex® tube with three slots in it in which 'doors' fit. Two of the slots were on the top of the tube 5cm apart. The third was placed between the two top slots 1cm from the slot closest to the reward. An artificial flower was placed at the entrance to the apparatus from the foraging chamber to encourage bees to enter the apparatus during pretraining and there was a reward chamber at the exit of the tube. The reward chamber consisted of a 5 x 5 x 5cm Perspex® box with a hinged lid. Two caps from 7ml plastic microtubes (Axygen®,Union City, CA) were placed in the reward chamber that could be filled with sucrose solution throughout testing.

The doors used during testing consisted of a metal door that could not be opened that was used to prevent bees from exiting the tube during testing, and a door to the reward chamber that was constructed from white plastic coffee cup lids (SOLO®). Doors were sized so that they

would fit smoothly into the slots in the tube, but did not form a perfect fit with the surface of the tube.

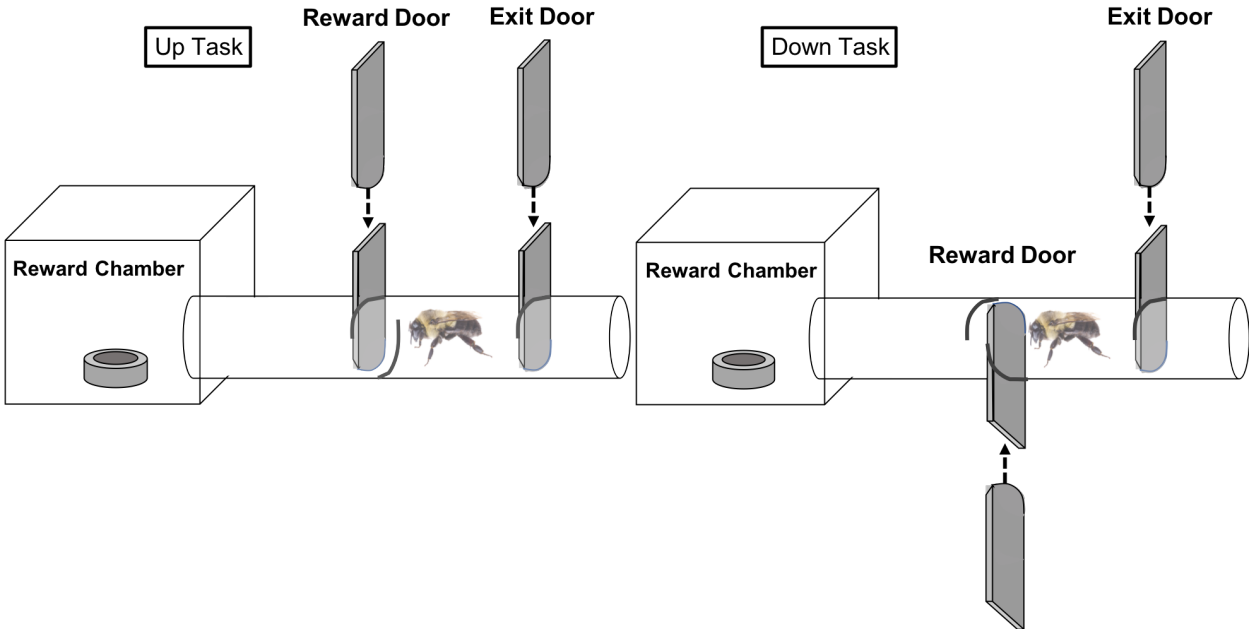

Figure 3-1 Apparatus. The apparatus consisted of a tube attached on one end to a foraging chamber and on the other to a reward chamber. The reward chamber contained a sucrose reward. In each trial two doors were inserted into the tube, a metal door that the bee could not open, and a plastic door that the bee could open. *Up task* trials required bees to lift the plastic door out of the tube to access the sucrose reward. *Down task* trials required bees to depress the door to access the sucrose rewards.

3.2.1.2.1 Up task

For *up task* trials the doors were inserted into the two slots at the top of the tube. The metal door that was placed in the slot nearest to the foraging chamber to prevent the bees from leaving the apparatus, and the plastic door was inserted into the slot nearest to the reward chamber.

3.2.1.2.2 Down task

In the *down task*, the metal door was inserted into the slot preventing exit from the apparatus. The plastic door was inserted into the slot on the bottom of the tube and held in place with a resistance mechanism. The resistance mechanism consisted of the plastic door affixed to

two clear plastic pieces outside the tube that extended 4cm in either direction down the length on the tube. The clear plastic extensions were inserted into two loops of clear plastic attached to the underside of the tube. This design resulted in the door being held in place in the tube with resistance. The resistance was calibrated through trial and error such that the door would not move unless bees put pressure on it and pushed it down, but that the pressure needed was such that bees could successfully move the door.

3.2.1.3 Pre-training

During pre-training all bees in the colony had 24/7 access to the apparatuses. The artificial flowers at the entrances to the apparatuses and the reward chambers were baited throughout pre-training with 40% sucrose solution. The colony was given one week of pre-training before testing began. Following the initial week of pre-training the testing sessions began. The apparatuses remained accessible and baited with 40% sucrose when testing was not in progress to allow new foragers to complete the pre-training throughout the course of the experiment.

3.2.1.4 Testing procedure

Immediately prior to each testing session bees were observed pre-training and bees regularly making trips to the apparatus were selected for testing and tagged. The bees were then given time to deplete the sucrose reward at the entrance to the apparatus which then required bees to travel fully through the apparatus to the reward chamber to receive a 40% sucrose reward. During testing sessions only the bee that was currently being tested was given access to the apparatus, other bees were prevented from entering by inserting the metal door when they attempted to enter apparatus.

At the start of a testing session the plastic door was inserted into the tube and the metal door was inserted after the bee being testing entered the apparatus. Trials started when the bee was in the tube and the metal door was in place. Trials ended either when the bee opened the plastic door or 300s had passed. If the bee opened the plastic door it was allowed to enter the reward chamber and fill to repletion on 40% sucrose. If the bee failed to open the plastic door then the plastic door was opened by the experimenter and the bee was then allowed to collect sucrose reward. In trials where it was necessary for the experimenter to open the plastic door the experimenter waited until the bee was oriented away from the plastic door before opening it to avoid interfering with the bee's learning on the task. After bees filled to repletion on the sucrose reward the metal door was removed from the apparatus and the bees were allowed to return to the colony to deposit nectar.

Each testing session consisted of 10 trials of both the *up task* and the *down task*. The trials were structured in a block design with 10 trials of one trial type completed consecutively followed by 10 trials of the second trial type. The order in which the trial types were completed was counterbalanced across bees. All trials were completed in a single session, with bees self-initiating trials for 20 consecutive trials.

All trials were video recorded for subsequent analysis using a HERO3 video camera (GoPro Inc., USA).

3.2.1.5 Video scoring

All videos were scored using Observer XT software. The videos were scored for latency to success, time spent pushing on the plastic door, time spent inverted while in contact with the plastic door, time spent pushing on the exit, and time spent inverted while in contact with the metal door. Latency was measured from the time the doors were inserted until the bee had 50%

of its body underneath the door. If bees were unsuccessful at opening the door then they were given the max trial time (300s). Pushing, whether in relation to the plastic or metal door, was operationally defined as making contact with the door with either legs or head cap while upright. Inverting, for both the plastic and metal door, was defined as a bee having more than 50% of its underside exposed while in contact with the door. All behaviours were scored as mutually exclusive and behaviours engaged in while not in contact with a door were not scored.

3.2.1.6 Data analysis

Latency scores for each trial were used to calculate proportion scores from total inverting and pushing times for each trial.

All data was analyzed by conducting repeated measures ANOVA in IBM® SPSS®. Additional analysis of the relations between dependent variables was conducted in RStudio® using rmcorr: Repeated Measures Correlation (Bakdash & Marusich, 2017).

3.2.2 Results

All 20 bees successfully completed 10 trials of each trial type.

3.2.2.1 Latency

Latency to success for trials on the *up task* and *down task* were analyzed with a mixed ANOVA, trial was a within-subjects factor and start order was entered as a between-subjects factor. Start order was the label given to the order in which bees completed the two tasks. On the up task (Figure 3-2), bees significantly reduced their latency to success across trials, $F(3.38,60.85) = 16.34$, $p<.001$. There was also a significant effect of start order with bees that completed the *up task* first taking significantly longer to solve that task than those who completed it second, $F(1,18) = 23.59$, $p<.001$. There was also an trial X start order interaction, $F(3.38,60.85) = 3.03$, $p=.02$

Analysis of latency on the down task showed a significant effect of trial with latency reducing across trials, $F(2.47, 43.68) = 5.72$, $p=.004$ (Figure 3-2). There was a significant effect of start order, $F(1, 18) = 5.69$, $p=.03$, with those bees that completed the down trials second having shorter latencies than those completing down trials first. The trial X start order interaction was not significant, $F(2.47, 43.68) = 1.87$, $p = .158$.

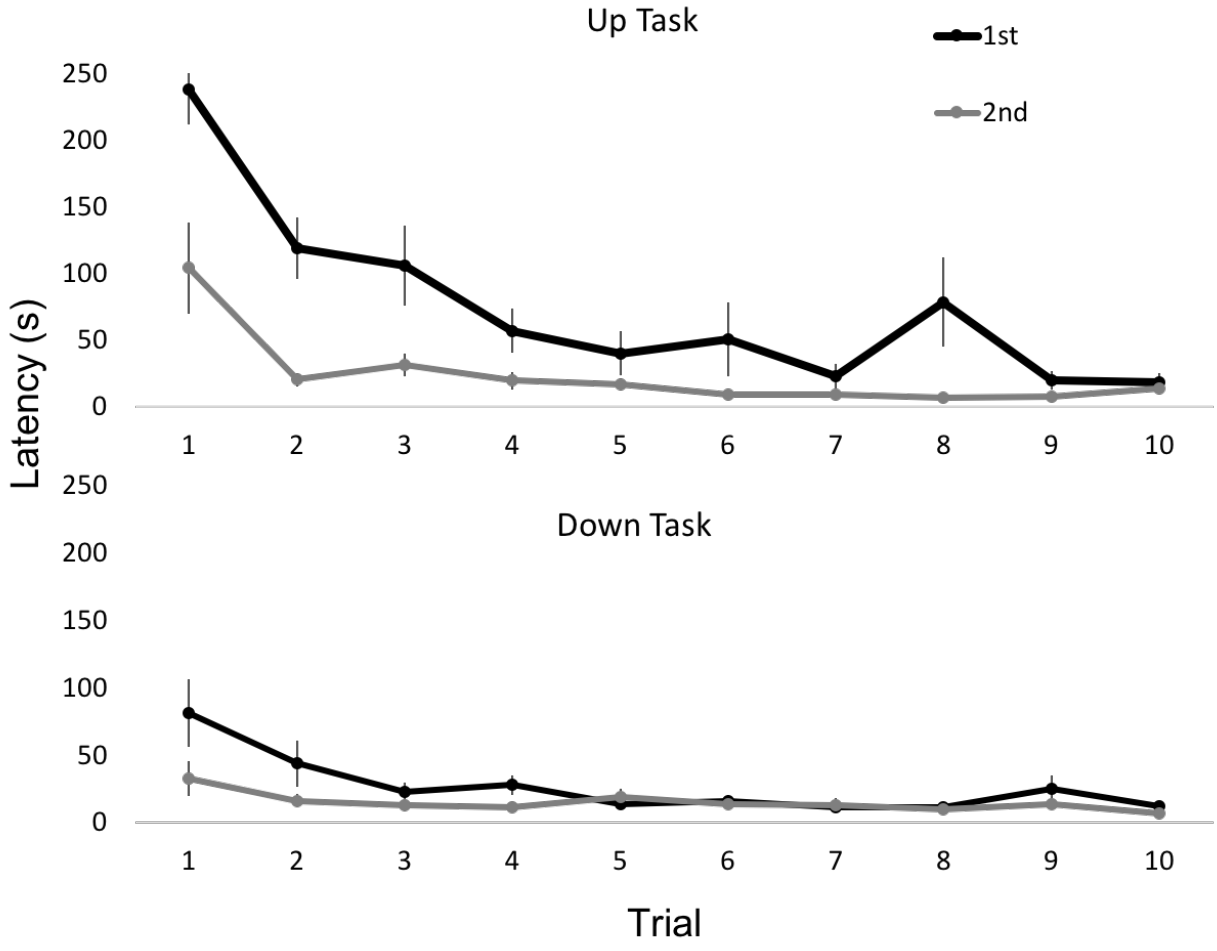

Figure 3-2 Experiment 1 latency. Trials on the *up task* are shown in the top panel. Bees reduced latency across trials on the *up task*, with bees that completed the *up task* second completing trials faster than bees that completed the task second. The bottom panel shows *down task* trials. Latency decreased across trials, and bees that completed the task second were faster than those that completed it first.

3.2.2.2 Time inverted

Proportion of time spent inverted for both the up task and the down task was analysed using a mixed-ANOVA with trial as a within-subjects factor and start order as a between-subjects factor.

There was a significant increase in proportion of time spent inverting across trials in the up task, $F(9, 162) = 5.65$, $p < .001$ (Figure 3-3). There was no significant effect of start order, $F(1, 18) = 3.34$, $p = .08$, nor was the trial X start order interaction significant, $F(9, 162) = .36$, $p = .951$.

Analysis of proportion of time inverted on the down task found no significant effect of trial, $F(3.096, 55.733) = 2.194$, $p = .097$, start order, $F(1, 18) = .689$, $p = .417$, or the interaction of trial X start order, $F(3.096, 55.733) = 1.497$, $p = .224$ (Figure 3.3).

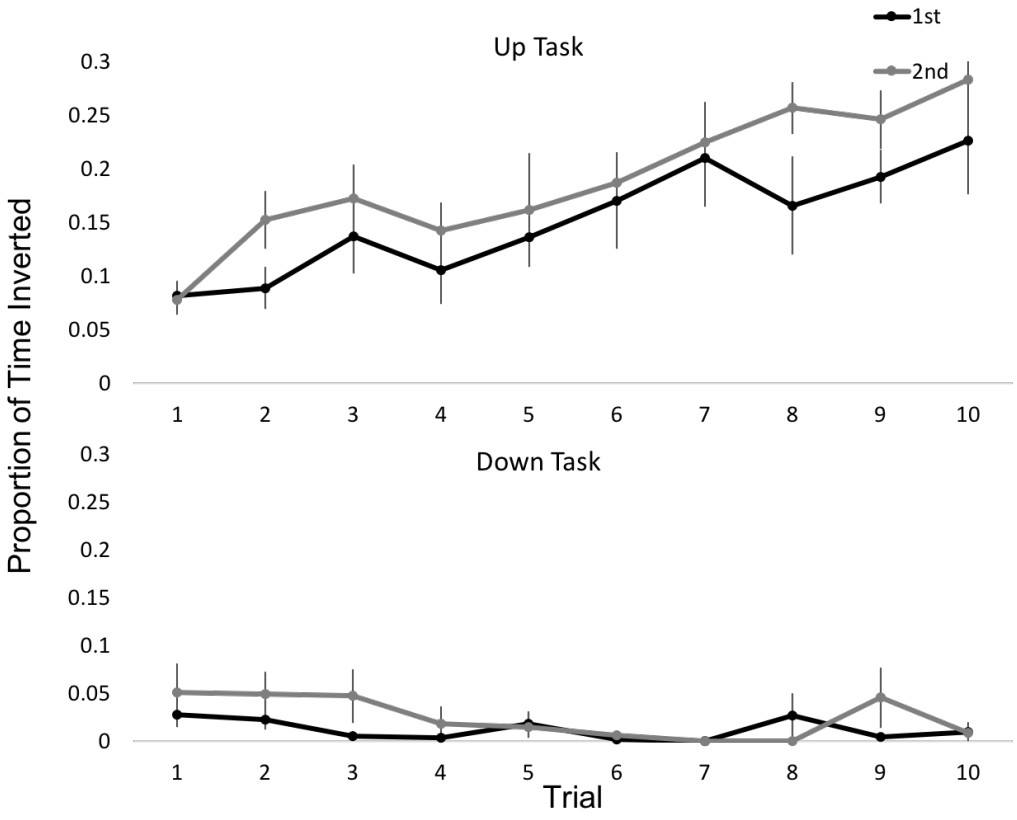

Figure 3-3 Experiment 1 time inverted. The top panel represents the time spent inverted on the *up task*. Bees increased the amount of time spent inverted across trials with no

difference between those bees that completed the task first and those that completed the task second. Time spent inverted on the *down task* is represented in the bottom panel. Bees did not change the amount of time that they spent inverted across *down task* trials and there was no effect of order.

3.2.2.3 Pushing

A mixed-ANOVA analysis of proportion of time pushing on the *up task* showed a significant effect of trial, $F(9, 162) = 3.54$, $p < .001$, but no significant effect of start order, $F(1, 18) = .819$, $p = .378$. There was a significant interaction between trial X start order, $F(9, 162) = 2.058$, $p = .036$. Bees increased time spent pushing in early trials and then reduced time spent pushing in later trials, but this pattern occurs more rapidly in bees that completed the up task second (Figure 3-4).

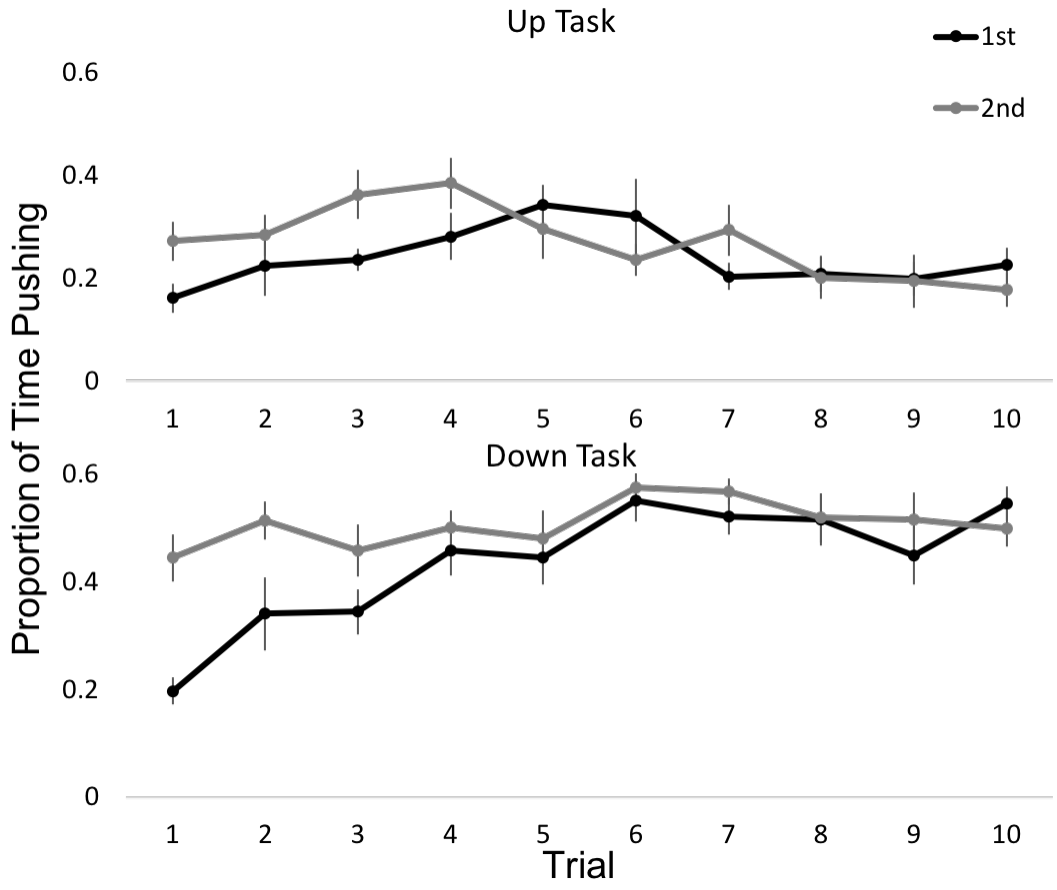

Figure 3-4 Experiment 1 time pushing. Time pushing on the *up task* is shown in the top panel. Bees significantly changed their time pushing across trials by increasing and then

decreasing time pushing Bees that completed the *up task* second showed more of an increase in time pushing in early trials than bees that completed the *up task* first. The bottom panel shows pushing on *down task* trials. Bees increased the time spent pushing across trials with the increase in early trials being larger in bees that completed the task second.

On the *down task*, bees increased their proportion of time spent pushing regardless of start order, but those bees that completed the down task first showed a greater change across trials than bees that completed the down task second. Analysis of proportion of time spent pushing on the down task found that trial, $F(9, 162) = 7.465$, $p < .001$, start order, $F(1, 18) = 4.473.54$, $p = .049$, and the trial X start order, $F(9, 162) = 2.574$, $p = .009$, were all significant.

3.2.2.4 Interaction with the Exit Door

The proportion of time pushing on the exit and time spent inverted at the exit were analyzed separately using mixed-ANOVA.

Proportion of time spent interacting with the exit while inverted declined across *up task* trials ($F(1.940, 34.915) = 5.352$, $p = .01$) (Figure 3-5). There was no significant effect of either start order ($F(1, 18) = .012$, $p = .914$), or the start X trial interaction ($F(1.940, 34.915) = 1.165$, $p = .323$). On the *down task,* the proportion of time spent inverted while interacting with the exit changed across trials ($F(9, 162) = 3.543$, $p < .001$). There was a significant effect of the trial X start order ($F(9, 162) = 2.058$, $p = .036$), but no significant effect of start order ($F(1, 18) = .819$, $p = .378$).

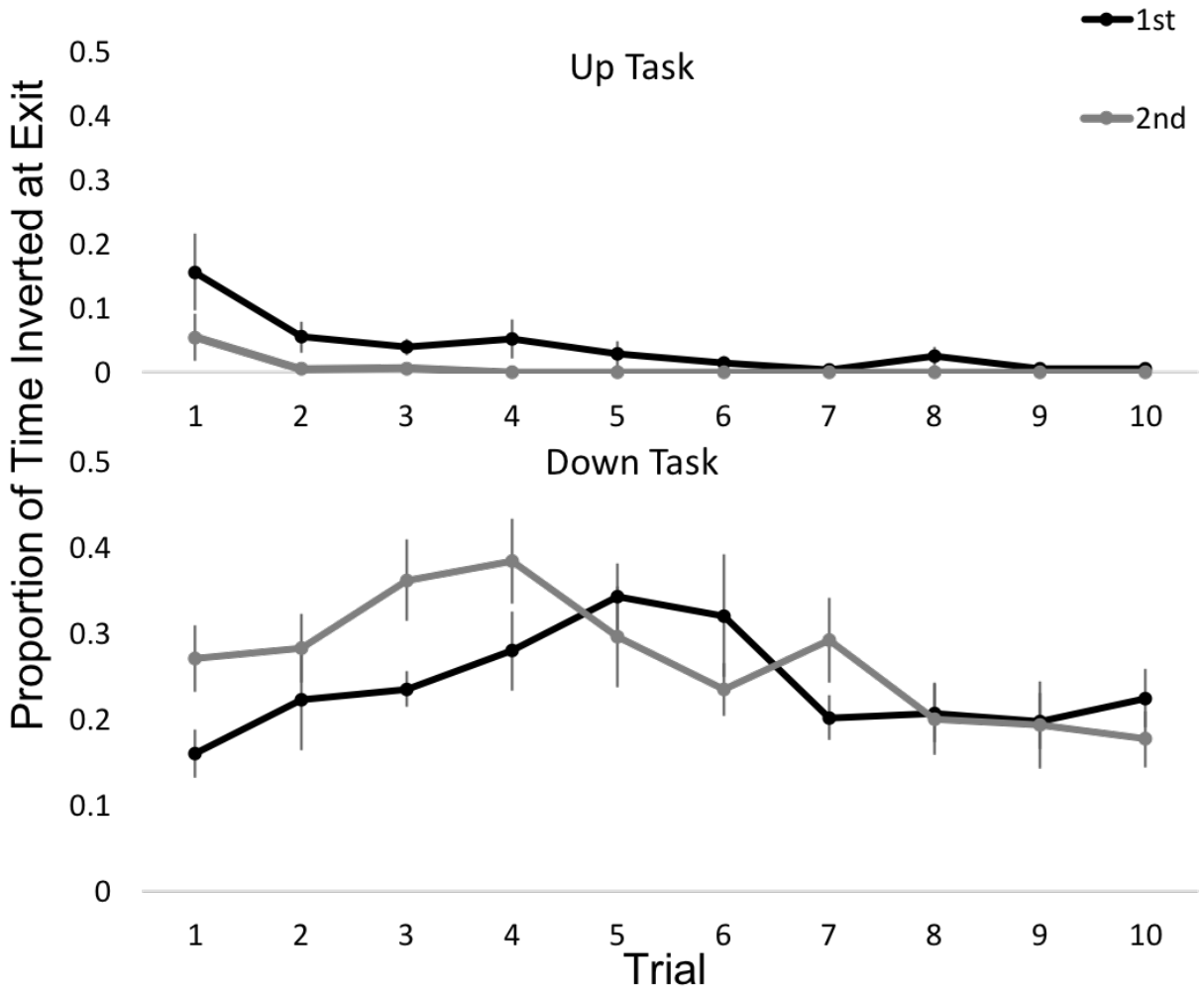

Figure 3-5 Experiment 1 time inverted at exit. The top panel shows a reduction in time spent inverted at the exit on the *up task*. There were no differences between bees that completed the *up task* first and those that completed it second. The bottom panel shows the change in time inverted at the exit in the *down task*. Bees increased their time spent inverted at the exit in early trials and then reduced time inverted at the exit in latter trials. This change across trials is more pronounced in bees that completed the *down task* second.

Proportion of time spent pushing on the exit door in the *up task* showed a significant change across trials ($F(2.922, 52.604) = 6.616$, $p = .001$), as well as a significant effect of start order ($F(1, 18) = 20.493$, $p < .001$) and a significant effect of the trial X start order interaction ($F(2.922, 52.604) = 2.855$, $p = .047$) (Figure 3-6). On the *down task*, proportion of time pushing on the exit reduced significantly across trials ($F(9, 162) = 3.543$, $p = .003$). There was also a

significant trial X start order interaction ($F(9, 162) = 2.058$, $p = .036$). Start order on its own was not significant ($F(1, 18) = .819$, $p = .378$).

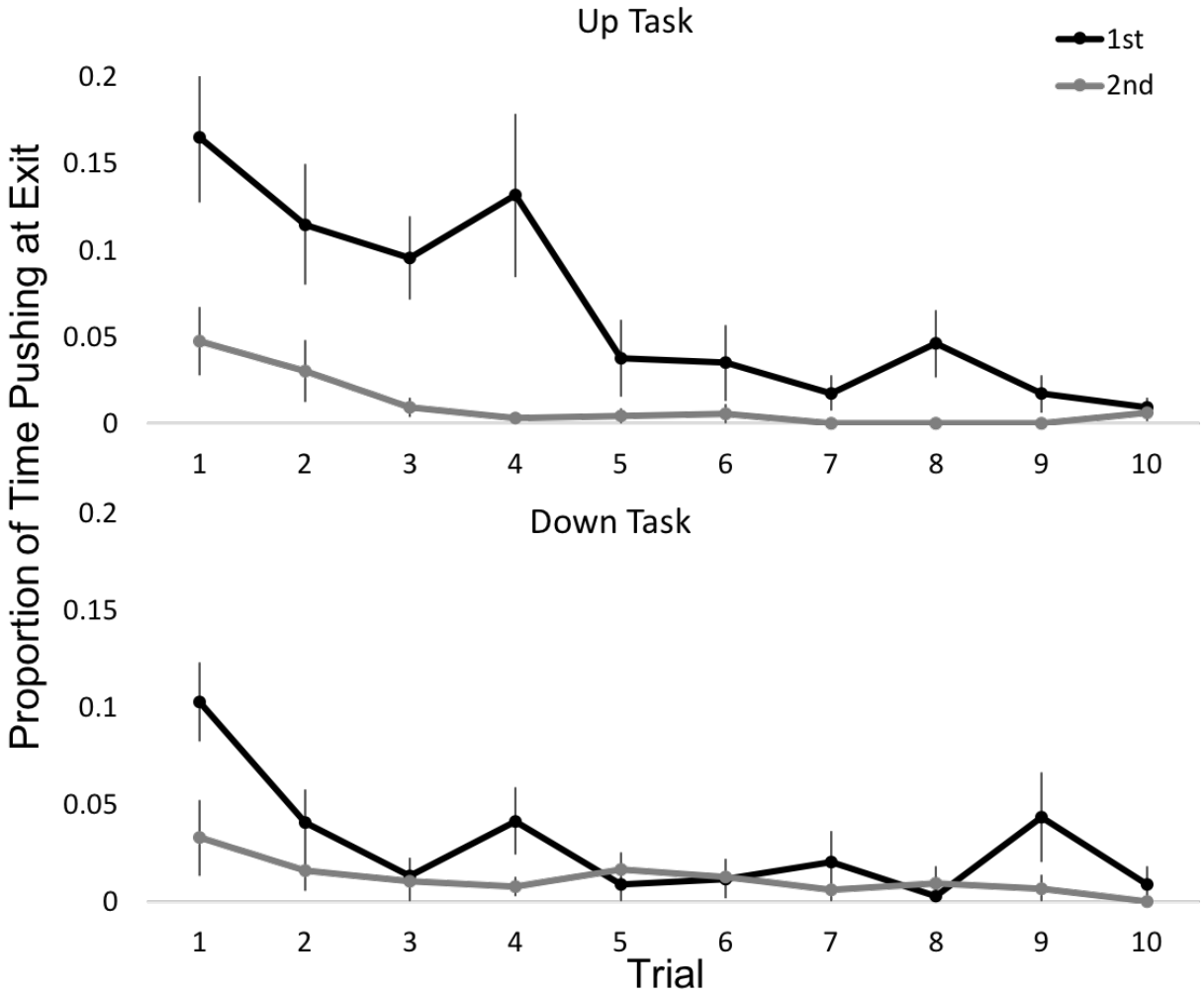

Figure 3-6 Experiment 1 time pushing at exit. Time spent pushing on the exit is shown in the top panel. There was a significant decline in pushing on the exit across trials. Bees that completed the *up task* first pushed on the exit more in early trials than bees that completed the *up task* second, but both groups pushed on the exit comparably in later trials. Proportion of time pushing on the exit during down trials is shown in the bottom panel. Bees reduced time pushing on the exit across trials, with bees that did the *down task* first pushing more in early trials than those that completed the task second.

3.2.2.2 Relations between dependent measures

Correlations between trial latency and two other dependent measures, proportion of time inverted and proportion of time pushing, were determined using repeated measures correlations

with the R package rmcorr: Repeated Measures Correlation (Bakdash & Marusich, 2017). Correlations for variables in the *up task* and *down task* were analyzed separately.

3.2.2.2.1 Up task

For the bees that completed *up task* first there was a significant correlation between latency and time inverted ($r(89) = -.323$, $p = .002$)(Figure 3-7) as well as between latency and time spent pushing ($r(89) = -.222$, $p = .034$)(Figure 3-8). For bees that completed *up task* second there was a significant relation between latency and time spent inverted ($r(89) = -.382$, $p < .001$)(Figure 3-7), but no significant relation between latency and time spent pushing ($r(89) = .063$, $p = .554$)(Figure 3-8).

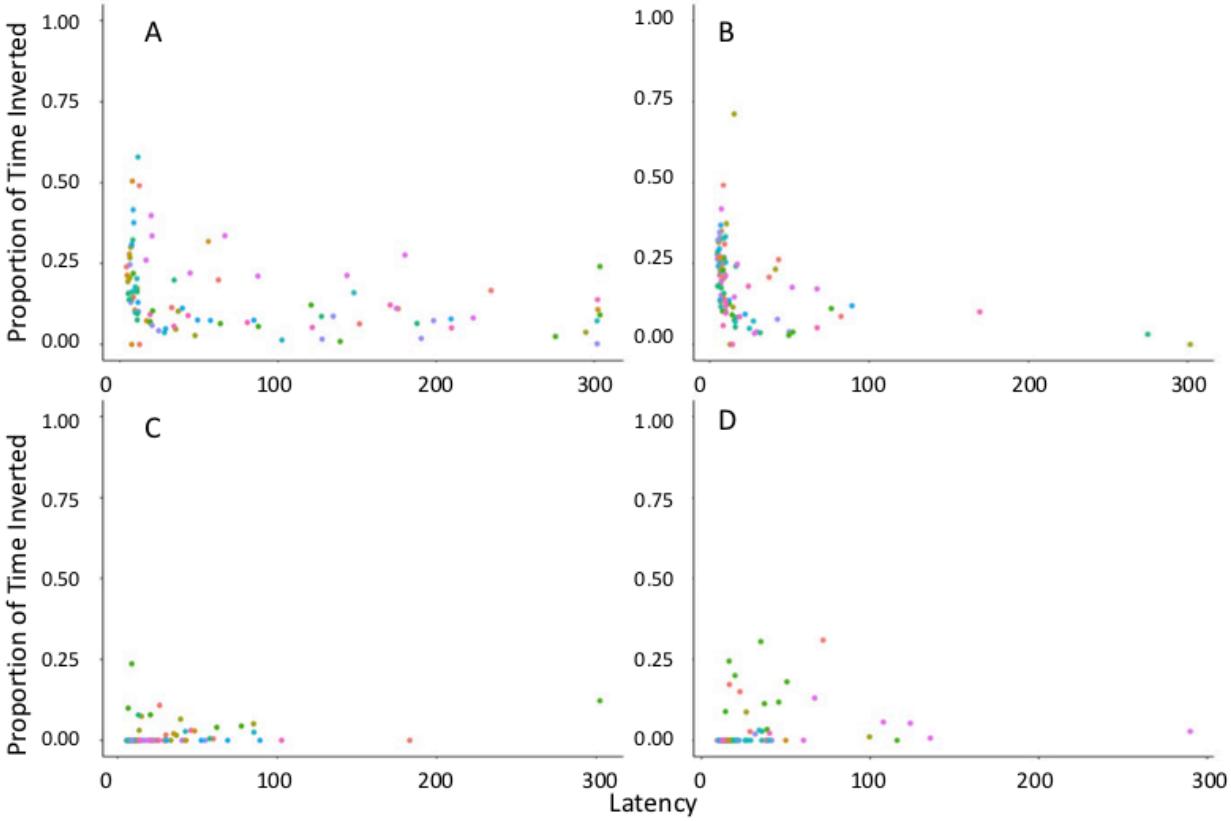

Figure 3-7 Experiment 1 relation between latency and time inverted. Panel A shows the significant correlation between latency and time inverted on the *up task* for bees that completed the *up task* first. Panel B shows the significant relation between latency and time inverted on the up task for bees that completed the *up task* second. Panel C shows the relation between latency and time inverted on the *down task* for bees that completed the

down task **first, which was not significant. Panel D shows the relation between latency and time inverted on the** ***down task*** **for bees that completed the** ***down task*** **second, which was not significant. In each panel the colours represent data from individual bees.**

3.2.2.2.2 Down task

Bees that completed the down task first showed a significant relation between latency and time spent pushing ($r(89) = -.536$, $p < .001$)(Figure 3-8), but not a significant relation between latency and time spent inverted ($r(89) = .189$, $p = .072$)(Figure 3-7). Bees that completed the down task second showed the same trend as those that completed the task first, a significant relation between latency and time pushing($r(89) = -.382$, $p < .001$)(Figure 3-8), but no relation between latency and time inverted ($r(89) = .097$, $p = .362$)(Figure 3.7)

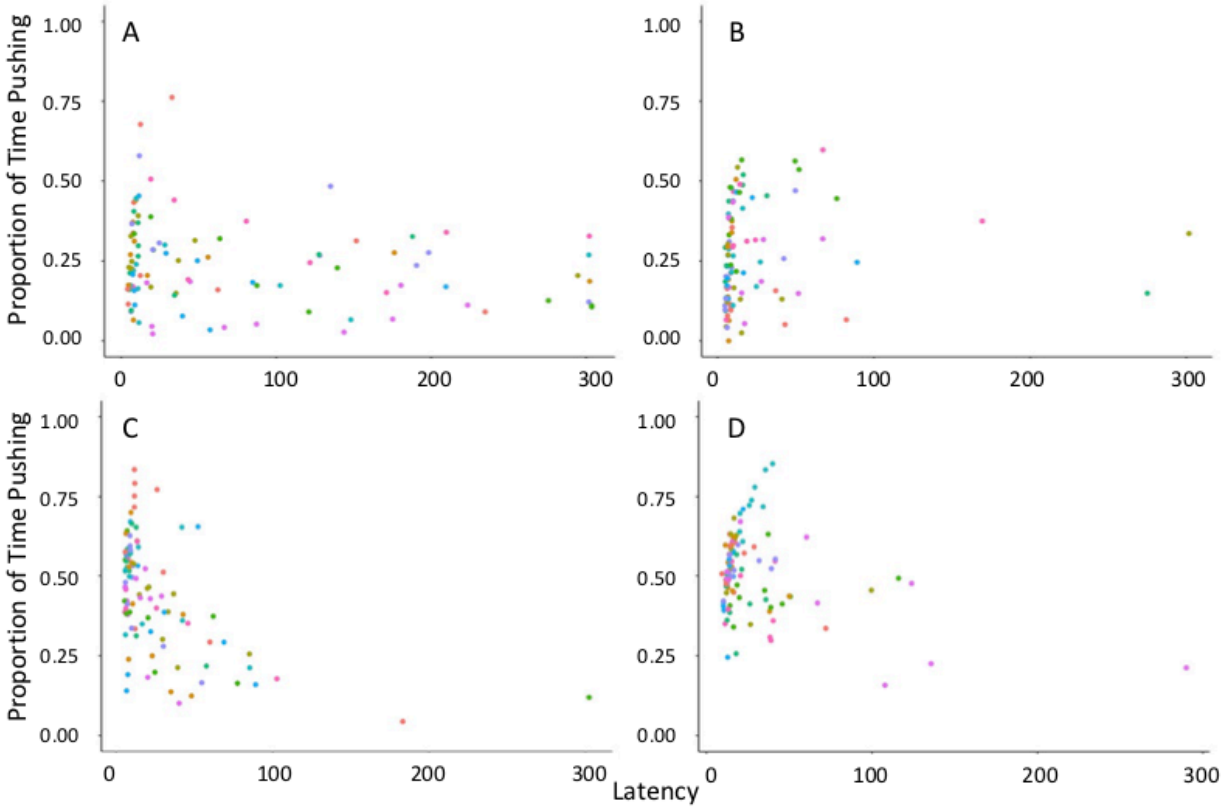

Figure 3-8 Experiment 1 latency and time pushing relation. There was a significant negative correlation between latency and time spent pushing on the ***up task*** **for bees that completed the** ***up task*** **first (Panel A), but that relation was not found in bees completing the** ***up task*** **second (Panel B). Panel C shows the significant correlation between latency and time spent pushing on the** ***down task*** **for bees that completed the** ***down task*** **first, and panel D shows the significant relation between latency and time pushing on the** ***down task*** **for bees**

completing the *down task* second. The colours present in each panel represent each individual bee.

3.2.3 Discussion

Bees improved performance across trials, as measured by reduced latency, on both the up task and the down task. This is consistent with findings in Chapter 1 for the up task and provides justification for use of the down task as a model for flower handling. A surprising finding was that bees learned a task faster when it was the second task learned compared to when it was the first task learned and this was the case for both the up and down tasks. This shows not only an absence of interference, but also facilitation. The two tasks used here were distinct in the motor behaviour required for the solution, but similar in that there was a door that had to be moved and the reward was in the same location within the apparatus. It is possible that rather than experiencing interference the bumblebees were able to extract information about the general task demands while completing the first handling task and apply that to the second handling task. Using general information about task demands and applying it to comparable problems is a frequent occurrence in animal cognition research and referred to as 'learning-to-learn'(Shettleworth, 1998).

The behaviour of bees at the exit door during testing can provide some information on what general task demands the bees may have learned. The proportion of time spent pushing on the exit door declined across trials on both the up task and the down task. Pushing on the exit door was an unsuccessful behaviour for both tasks, so extinction of exit door behaviours that occurred when bees were learning the first task could transfer to the second task. This does appear to have occurred, with bees in the pushing on the exit door significantly less often when they completed a task second. Therefore, the facilitation that occurred could be due to a transfer

of extinction training on unsuccessful behaviours in the first task that bees learned to the second task.

In light of the facilitation that occurred it is necessary to consider if the absence of interference effects could be due to the behaviour required to be successful on one task overlapping with the behaviour required to be successful on the second task. It has been suggested that an important factor in interference costs is the morphological similarities between flower species (Gegear & Laverty, 1995). Analysis of the behavioural data strongly suggests that this is not what occurred. On the up task bumblebees increased their proportion of time spent inverted, which is the successful strategy. This pattern in proportion of time spent inverted was not observed on down task trials, indicating that changes in inverting behaviour was specific to the up task. With regard to pushing behaviour, the successful strategy for the down task, bees increased their time across trials for the down task, but did not show an increase across trials on the up task. This again demonstrates that reinforcement of pushing behaviour was specific to the down task. The correlation between increased use of the successful behaviours for each task and reductions in latency for task adds even more support to distinct behaviours being reinforced for each task. The data then clearly shows that overlap in the specific required motor pattern for each task is not responsible for the absence of interference.

Although inverting was successful exclusively for the up task and pushing was successful exclusively for the down task, both behaviours occurred in both conditions. This supports the hypothesis developed in Chapter 2 that motor patterns used by bees during flower handling are innate. That is the motor patterns are not learned, but rather are instinctively initiated in a foraging context when the bees encounter an impasse, such as a petal, while trying to extract nectar. In this particular experiment bees engaged in a series of innate motor behaviours when

they encountered the door in the apparatus, which included both inverting and pushing on the door. On the up task inverting was successful, reinforced, and increased in frequency. On the down task pushing was successful, reinforced, and increased in frequency. Therefore, the learning that occurred on these tasks consisted of changes in the frequency of innate behaviours, not the establishment of novel behaviours.

3.3 Experiment 2 – Repeated Task Switching on a Flower Handling Task

In Experiment 1 bees were required to complete a single task switch. They acquired an initial flower handling task and then had to learn a second handling task. This design allowed for observation of interference on the acquisition of flower handling techniques, however it did not capture interference that occurs if a bee switches repeatedly while foraging. Experiment 2 was designed to measure the behaviour of bumblebees on a task requiring repeated switching, which would be representative of a foraging strategy without any constancy. In the experiment bees were required to repeatedly switch back and forth between two flower handling techniques. The goal of the experiment was to characterize any costs of the increased rate of switching as well as behavioural changes associated with those costs.

3.3.1 Method

3.3.1.1 Subjects and housing

Subjects were 20 bumblebees (*Bombus impatiens*) from the same colony provided by Biobest Canada Ltd. (Leamington, ON) that was used in Experiment 1. Housing conditions were identical to those used in Experiment 1. Tagging procedures were identical to those used in Experiment 1.

3.3.1.2 Apparatus

The apparatus was identical to that used in Experiment 1 (Figure 3-1).

3.3.1.3 Pre-training

The pre-training procedures established in Experiment 1 continued throughout Experiment 2.

3.3.1.4 Testing procedure

Individual trials, both for the *up task* and *down task*, were run identically to those in Experiment 1.

Bees completed 5 trials of each trial type (i.e. *up task* and *down task*) for a total of 10 trials. Trial type was switched after each successful trial. If bees were unsuccessful on a trial they continued to complete trials of that type until a successful trial before switching tasks. Where bees completed more than one trial before switching tasks, the first trial on the task was used for data analysis. The order in which bees completed the trials was counterbalanced across bees with five bees starting on the *up task* and five bees starting on the *down task*. All trials were completed in a single session.

All trials were video recorded for subsequent analysis using a HERO3 video camera (GoPro Inc., USA).

3.3.1.5 Video scoring

All videos were scored using Observer XT software. The videos were scored for latency to success, time spent pushing on the plastic door, time spent inverted while in contact with the plastic door, time spent pushing on the exit, and time spent inverted while in contact with the metal door. Behaviours were operationalized identically to Experiment 1.

3.3.1.6 Data analysis

As in Experiment 1, proportion scores were calculated from inverting and pushing times.

In instances where bees were unsuccessful on a trial and completed additional trials on the same task the data were analyzed for only the first trial.

All data was analyzed with repeated measure in IBM® SPSS®.

3.3.2 Results

All 20 bees completed 10 trials, 5 trials on each task. Video recording errors occurred for two bees which resulted in their exclusion from analyses. There were three instances in which bees were unsuccessful on the *up task*. In these three instances it was the bees' first *up task* trial, one of those bees having completed a *down task* trial prior to the *up task* trial, and they were successful on their second trial.

The trials were initially analyzed as an entire session, with both *up task* and *down task* trials included, and then subsequently analyzed by comparing performance on *up task* trials and *down task* trials across the two different start orders.

3.3.2.1 Session analysis.

3.3.2.1.1 Latency

A mixed-ANOVA, with start order as a between-subjects factor and trial as a within-subject factor, showed a significant drop in latency across trials ($F(3.562, 64.117) = 6.838$, $p < .001$) (Figure 3-9). There was also a significant trial X start order interaction ($F(3.562, 64.117) = 3.526$, $p = .001$), but no significant main effect of start order ($F(1, 18) = 1.390$, $p = .254$).

3.3.2.1.2 Inverting

Mixed-ANOVA results for proportion of time inverted showed a significant effect of trial, ($F(4.407, 70.506) = 2.927$, $p = .023$), and of the trial X start order interaction, ($F(4.407, 70.506) = 15.481$, $p < .001$) (Figure 3-10). There was no main effect of start order, ($F(1, 16) = .658$, $p = .429$).

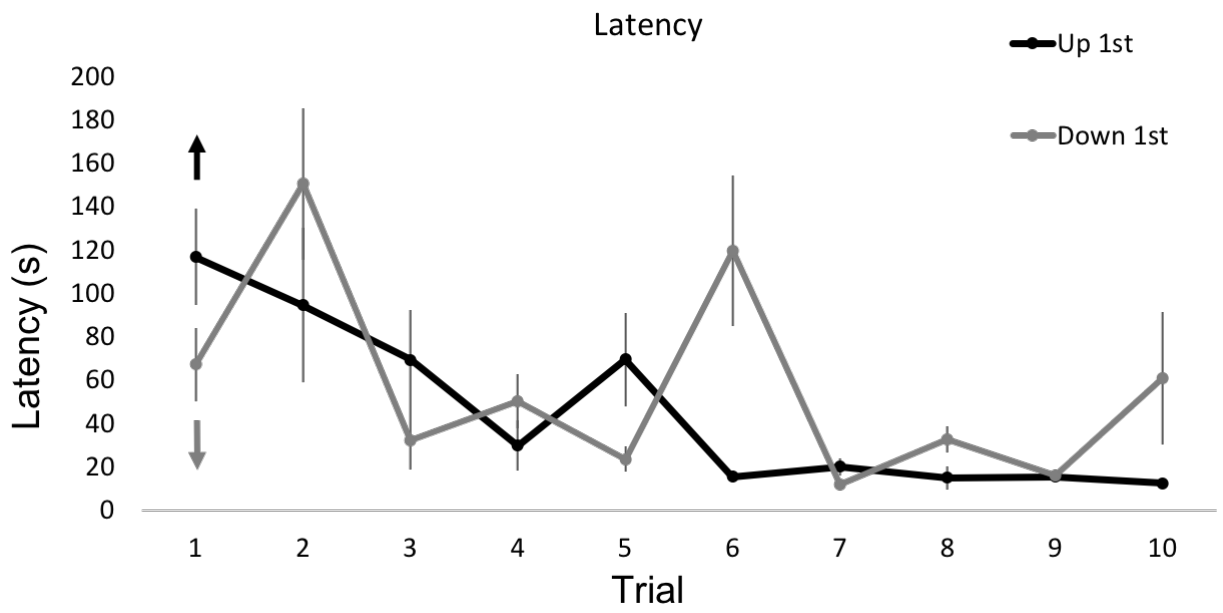

Figure 3-9 Experiment 2 latency. Bees significantly reduced latency across trials. There was a significant interaction between which task the bees completed on their first trial (as indicated by the arrows) and the change in latency across trials.

3.3.2.1.3 Pushing

Proportion of time spent pushing showed a significant effect of trial, ($F(9, 144)$ = 7.88, p < .001), and a significant trial X start order interaction, ($F(9, 144)$ = 15.538, p < .001), but no main effect of start order, ($F(1, 16)$ < .000, p = .998) (Figure 3-11).

3.3.2.1.4 Exit

There was a significant effect of trial for proportion of time spent interacting with the exit while inverted, ($F(3.236, 51.771)$ = 2.792, p = .046), but neither the start order X trial interaction, ($F(3.236, 51.771)$ = 1.958, p = .128), nor the main effect of start order, ($F(1, 16)$ = .002, p = .968), were significant (Figure 3-12). Analysis of the proportion of time spent pushing showed a significant effect of trial, ($F(4.038, 64.609)$ = 6.150, p < .001), a significant trial X start order interaction, ($F(4.038, 64.609)$ = 5.204, p = .001), and a significant effect of start order, ($F(1, 16)$ = 8.537, p = .01) (Figure 3-12).

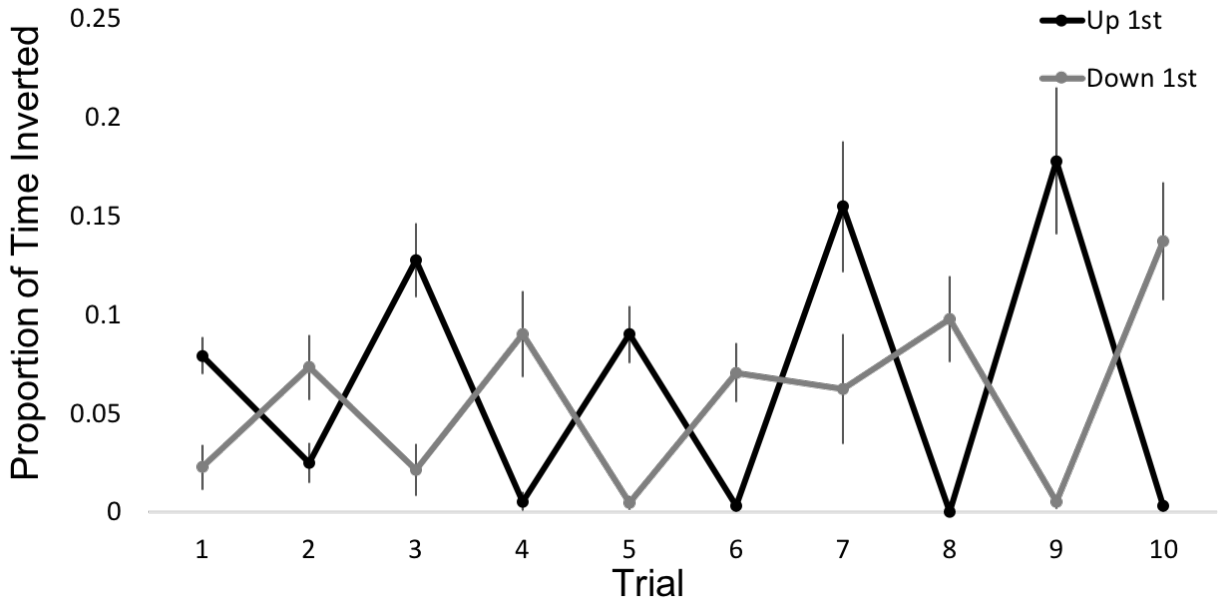

Figure 3-10 Experiment 2 time inverted. The proportion of time inverted changed across trials and there was a significant interaction between time inverted and the task on which bees started their testing session. Bees spent more time inverted on *up task* trials than on *down task* trials. This relation between trial type and time inverted results in bees that started on opposite tasks showing opposite patterns of time inverted across trials.

3.3.2.2 Task analysis.

3.3.2.2.1 Up task

There was a reduction in latency across up task trials ($F(2.334, 42.006) = 8.62$, $p < .001$), but no effect of start order ($F(1, 18) = 1.980$, $p = .176$) or interaction($F(2.334, 42.006) = .908$, $p = .425$) (Figure 3-13). There was a significant increase in time spent inverted across trials ($F(2.608, 41.732) = 3.682$, $p = .024$), but, again, no effect of start order ($F(1,16) = 4.021$, $p = .062$) or the trial X start order interaction ($F(2.608, 41.732) = .321$, $p = .783$) (Figure 3-14). Time spent pushing increased significantly across trials ($F(4, 64) = 6.591$, $p < .001$) and there was a significant interaction between trial and start order ($F(4, 64) = 5.247$, $p = .001$). However start order was not significant on its own ($F(1,16) = 1.767$, $p = .202$) (Figure 3-15). There was a

significant reduction in interaction with the exit across trials when measured as time inverted interacting with the exit (*F(2.1, 33.66)* = 4.532, *p* = .017) and time pushing on the exit (*F(2.375, 38.01*) = 8.619, *p* < .001) (Figure 3.16). There were no significant trial X start order interactions for either inverted at the exit (*F(2.1, 33.66)* = .271, p = .775) or pushing on the exit (*F(2.375, 38.006)* = .711, *p* = .521). Start order was significant for pushing on the exit (*F(1, 16)* = 5.708, *p* = .03), but not for time inverted at the exit (*F(1,16)* = .162, *p* = .693).

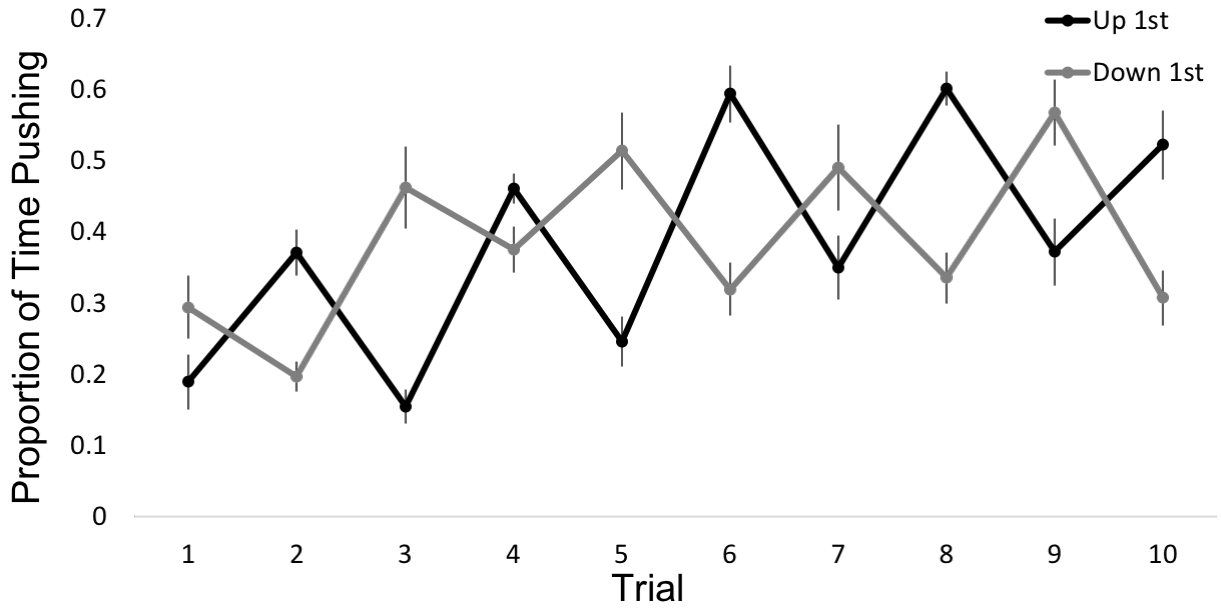

Figure 3-11 Experiment 2 time pushing. Bees increased the proportion of time pushing across trials. Bees spent more time pushing on down trials than on up trials, which resulted in opposite patterns of time pushed across trials for bees that started on different tasks and a start order by trial interaction.

3.3.2.2.2 Down task

Bees improved performance across down trials as demonstrated by a reduction in latency (*F(1.390, 25, 028)* = 8.341, *p* = .004), however there was no effect of start order (*F(1, 18)* = .128, *p* = .725) or the interaction between start order and trial (*F(1.390, 25.028)* = .516, *p* = .538) (Figure 3-13). Bees showed no significant change in proportion of time inverted across trials (*F(2.069, 33.108)* = 2.908, *p* = .067) and there was no effect of start order (*F(1, 16)* = 2.663, *p* =

.122) (Figure 3-14). However, there was a significant start order X time inverted interaction (*F(2.069, 33.108)* = 3.586, *p* = .038). Bees did change the amount of time they spent pushing across down task trials (*F(3.118, 49.88)* = 10.098, *p* < .001), but there was no effect of start order (*F(1,16)* = 1.324, *p* = .267) or a start order X trial interaction (*F(3.118, 49.888)* = 1.205, *p* = .318) (Figure 3-15). Bees' time spent inverted interacting with the exit did not change across trials (*F(1.160, 18,563)* = 3.113, *p* = .089), nor was there an effect of start order (*F(1, 16)* = .975, *p* = .338) or a start order X trial interaction (*F(1.160, 18.563)* = .307, *p* = .620) (Figure 3-16). Bees did reduce the amount of time they spent pushing on the exit (*F(2.328, 37.255)* = 5.788, *p* = .005), but there was no effect of start order (*F(1, 16)* = 3.985, *p* = .063) or the interaction between start order X trial (*F(2.328, 37.255)* = 1.125, *p* = .342) (Figure 3-16).

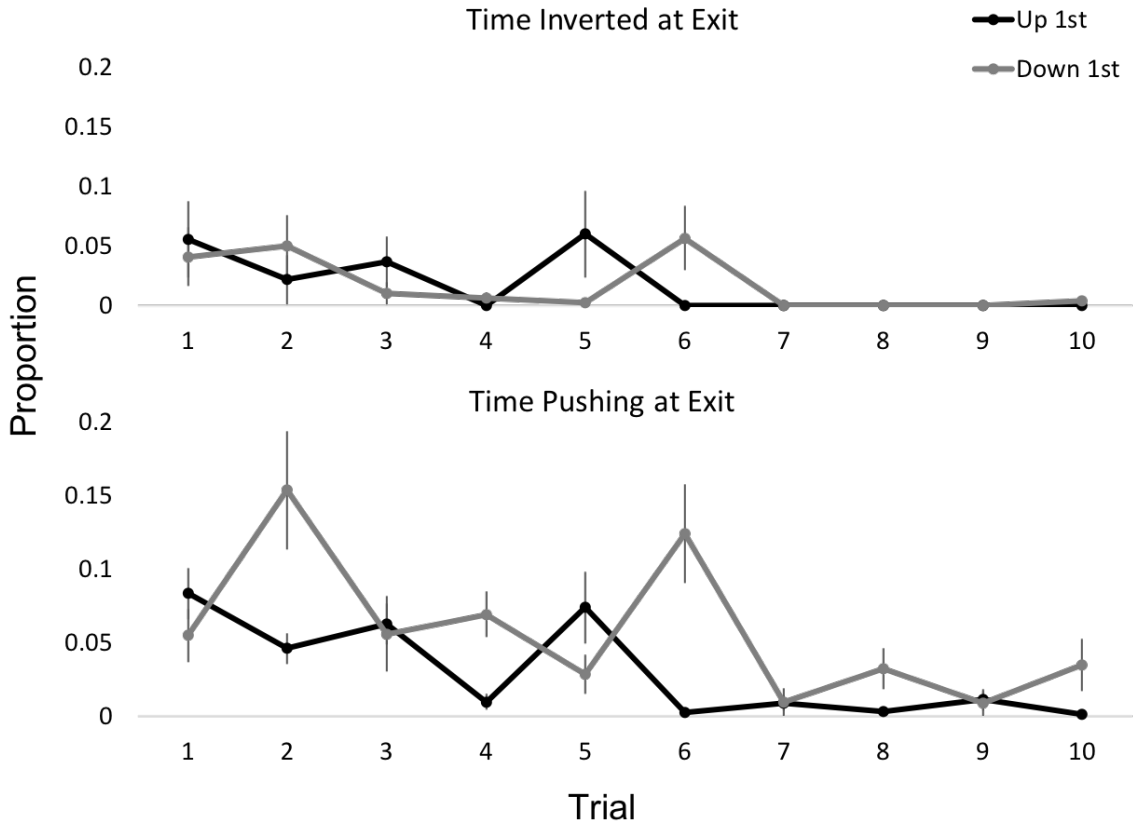

Figure 3-12 Experiment 2 interactions with the exit door. The top panel shows that bees reduced time spent inverted at the exit across trials, and did so equivalently for both start

orders. The bottom panel shows the change in time pushing at the exit across trials. Although bees in both start orders reduced time pushing on the exit across trials the pattern was different in bees that started on the *down task* first such that they spent more time pushing on the exit during up task trials than bees that experienced the *up task* first.

3.3.3 Discussion

Experiment 2 measured the interference that occurred when bumblebees were required to switch repeatedly between two different flower handling tasks. Bees were successful on both of the flower handling tasks and improved efficiency across trials on both tasks. When the data was analyzed as a session, with both the up task and down task trials included, there was a significant difference between those bees that started on the up task and bees that started on the down task.

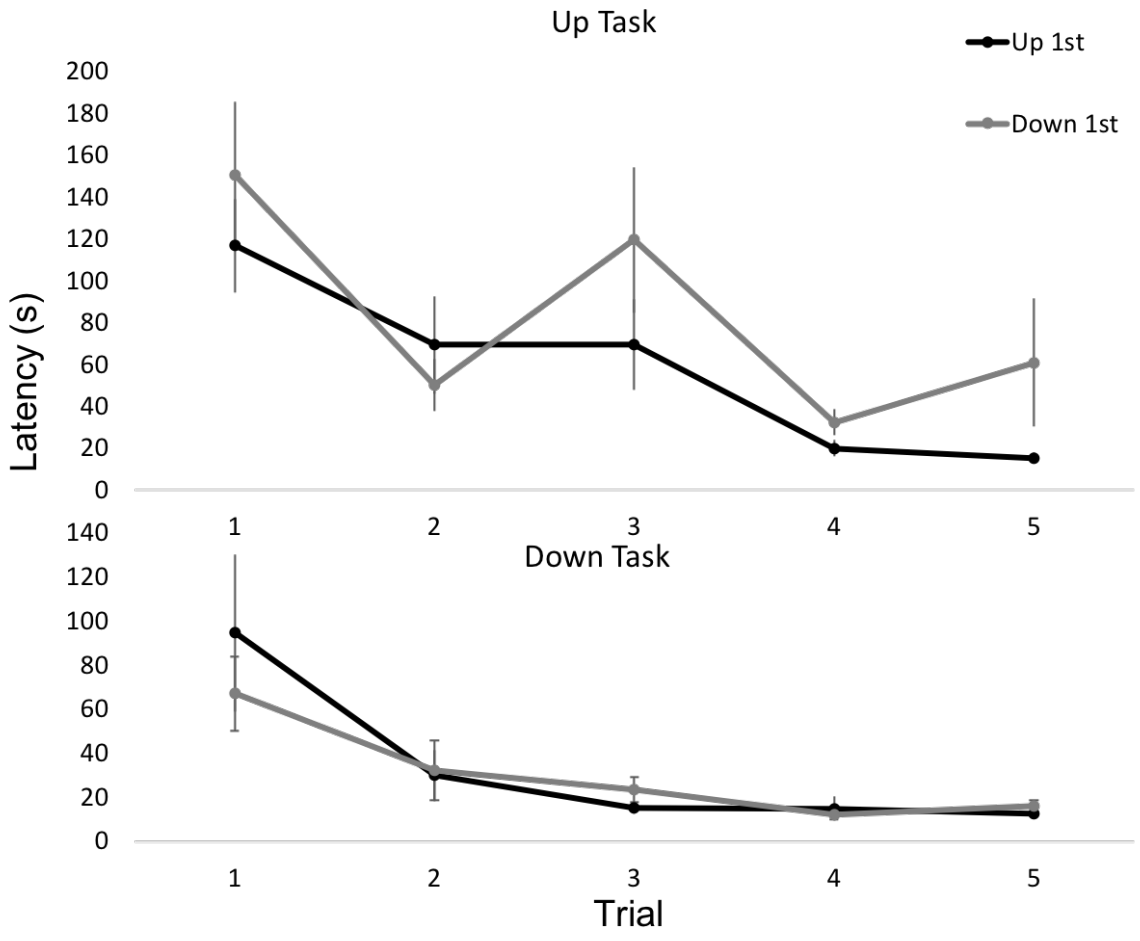

Figure 3-13 Experiment 2 latency separated by task. The top panel shows a significant reduction in latency across only the *up task* trials. The order in which bees completed trials

throughout the session did not affect the latency trend. The bottom panel shows a significant reduction in latency across only the *down task* trials. The order in which bees completed the trials did not significantly affect the trend.

However, this difference does not appear when trials of each particular type are isolated and analyzed. This suggests that the difference in latency across trials due to start order is a consequence of generally longer latencies for the up task than the down task, and therefore opposite alternating patterns in latencies for each start order.

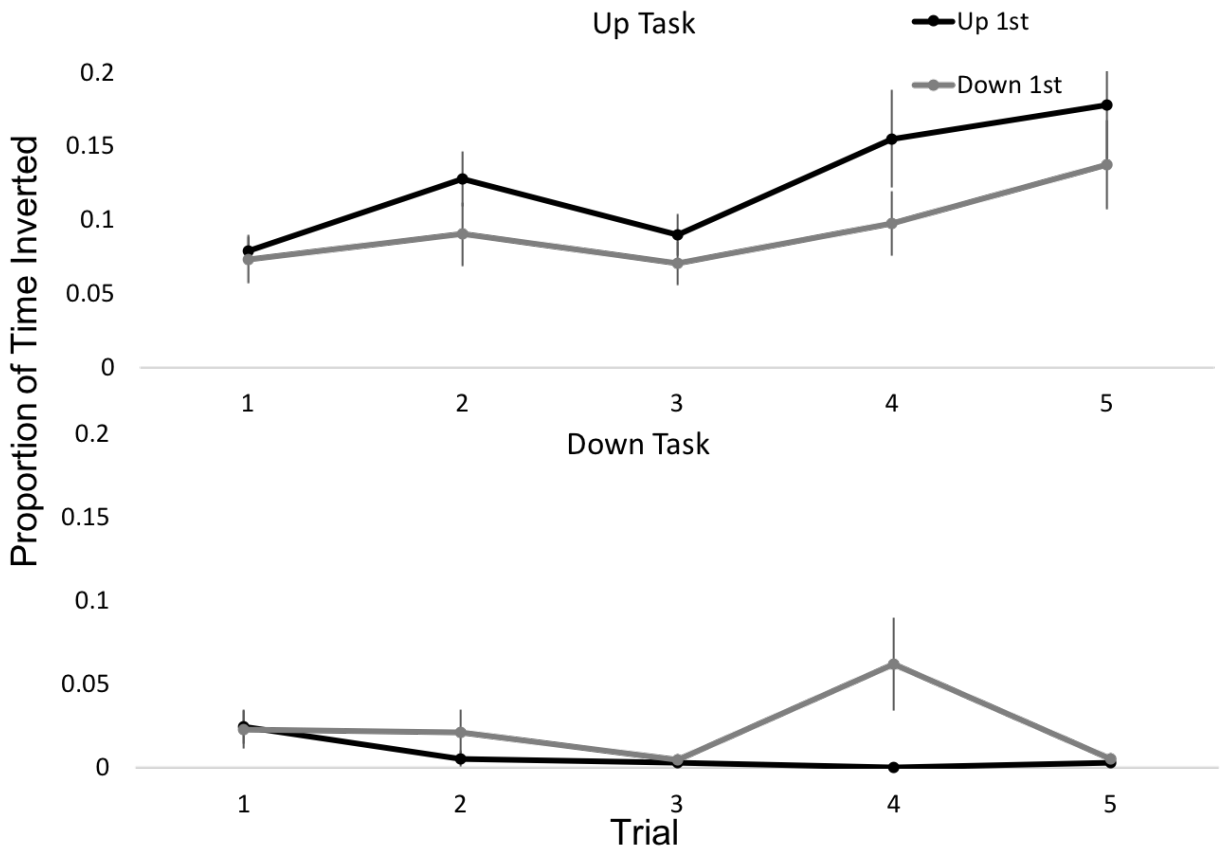

Figure 3-14 Experiment 2 time inverted separated by task. The top panel shows time spent inverted on only the *up task* trials. There is a significant increase in time inverted across trials, but that increase did not differ between the two start orders. The bottom panel shows time inverted on the *down task* trials. There was no change in time spent inverted on down trials for either start order.

As in Experiment 1, improvement on each of the flower handling tasks can be attributed to increased use of the successful strategy for that particular task (i.e. inverting for the up task,

pushing for the down task). The saw-toothed patterns that appears in the figures for time inverted (Figure 3.10) and time pushing (Figure 3-11), make clear that bees learned to specifically use the appropriate strategy when engaging in the task for which it was successful. This data shows no clear evidence for significant interference, in that bees were able to switch between the two handling techniques successfully.

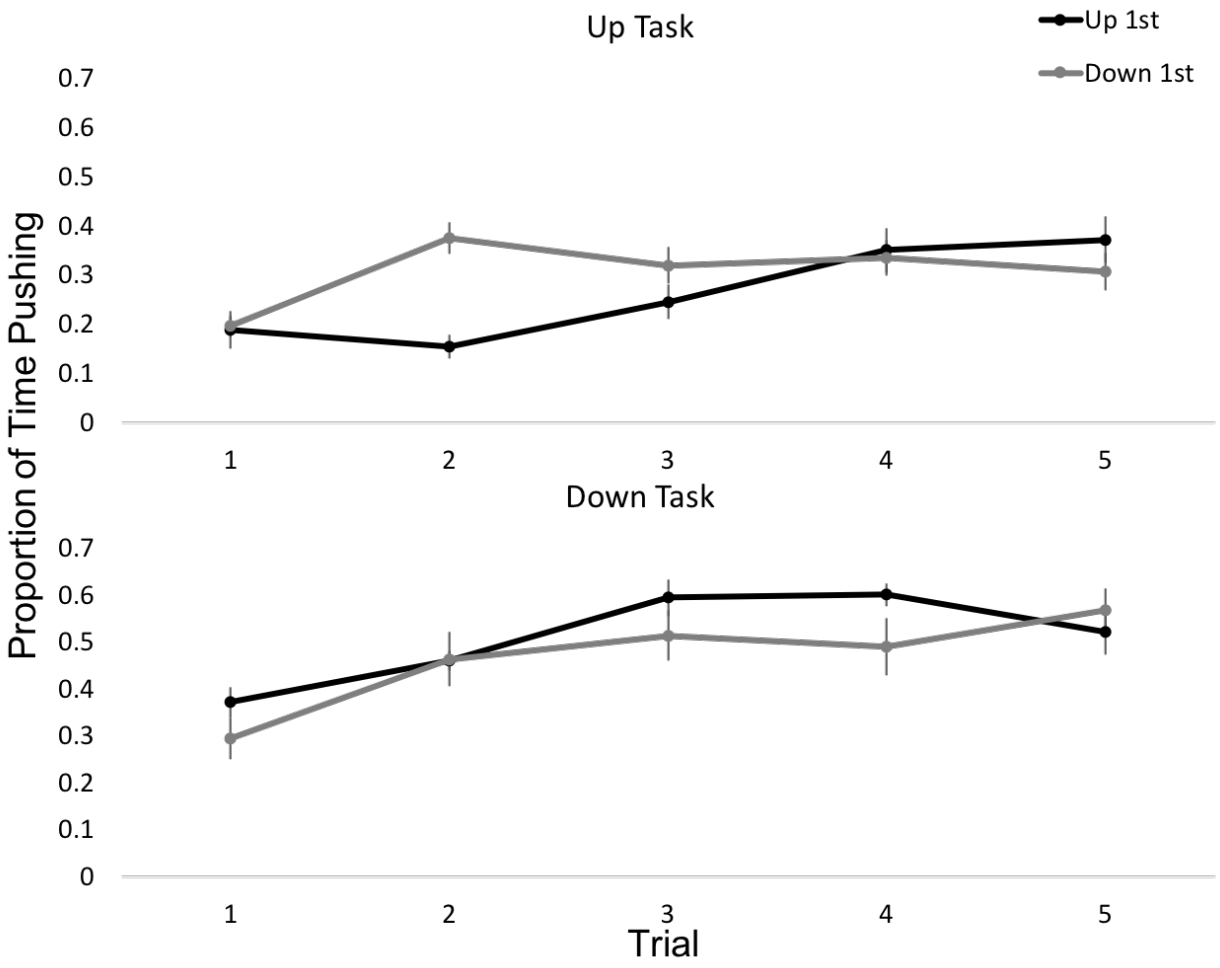

Figure 3-15 Experiment 2 time pushing separated by task. The top panel shows the significant increase in time pushing across the *up task* trials. There was an interaction between start order; bees that completed the *down task* first showed a spike in time spent pushing on *up task* trial 2 not seen in those bees that started with the *up task*. The bottom panel shows the increase in time spent pushing on the *down task* trials. There was no difference in the trend in pushing between the two start orders.

Bumblebees showed decreased interactions with the exit door across trials, despite switching repeatedly between the two tasks. This supports the observation in Experiment 1 that bees are able to extract information about general task demands and apply that information to both of the flower handling tasks tested here. It is possible that bees experienced interference in switching between the two flower handling techniques but that interference was obscured by facilitation from their learning general task demands.

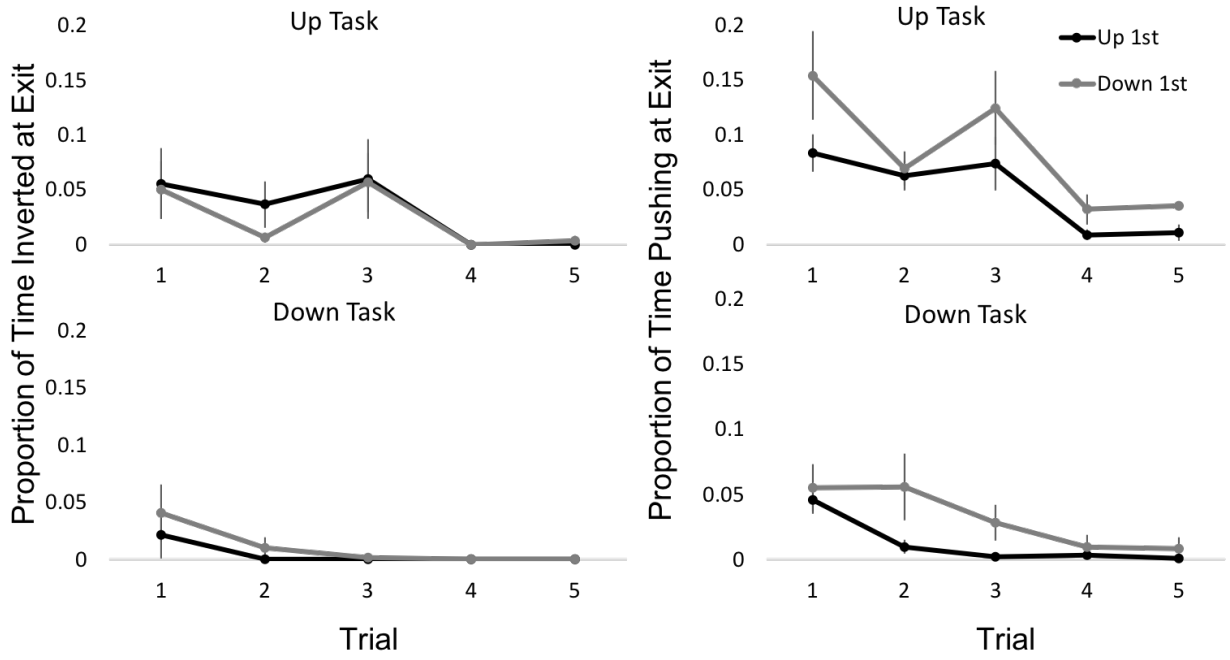

Figure 3-16 Experiment 2 exit behaviours separated by task. The top two panels show inverting and pushing behaviours at the exit door during *up task* trials. Both time inverted and time pushing decreased across trials. The elevated amount of time spent pushing by bees that experienced a down task trial first relative to those that experienced *up task* trials first was significant. The bottom panels show time inverted and time pushing on *down task* trials. There was no change in time spent inverted while interacting with the exit, but there was a reduction in time spent pushing on the exit across trials.

3.4 General Discussion

A long standing question in bumblebee foraging behaviour is why bumblebees limit their foraging to relatively few flower species, rather than foraging more flexibly on a wider variety of species (Waser, 1986; Chittka, Thomson, & Waser, 1999). Darwin (1876), introduced the hypothesis that learning more than one handling technique resulted in interference between the multiple techniques and a loss of foraging efficiency. It has since been shown that interference at the level of flower handling is not a likely explanation for flower constancy (Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998; Chittka, Thomson, & Waser, 1999), but a complete characterization of the mechanisms of switching behaviour of bees at the level of flower handling had not previously been undertaken. The results here support my hypothesis that when bees are switching between two handling techniques they are switching between two innate motor patterns, so bees are engaging in associative learning, rather than learning multiple novel motor patterns. This makes their switching on flower handling tasks similar to associative switching tasks (reversal and serial reversal learning) at which they are successful (Strang & Sherry, 2014). This hypothesis is consistent with previous observations on spatial reversal tasks in which bees incur switching costs at the level of associative learning, but not motor behaviour (Chittka & Thomson, 1997). Reliance on innate motor patterns for flower handling may be the mechanism responsible for the flexibility that allows bumblebees to function as generalist foragers as discussed in Chapter 2, and the same mechanism may serve to eliminate motor learning interference when switching between flower types.

3.4.1 Costs of Switching

Previous investigations of the interference hypothesis consistently found costs of switching, even though those costs were too low to be of ecological significance (Woodward &

Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998). Here there was no evidence of interference costs as bumblebees were capable of acquiring two motor tasks and switching successfully between the two. One possible explanation for the discrepancy is the different types of interference being measured. In previous work, interference costs were measured in memory, asking if bees could remember an initially learned flower handling technique after having learned a second technique (Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998). Instead of focusing on already heavily studied memory interference costs of switching, Experiment 1 explored costs of switching during acquisition. It is possible that bumblebees do not incur any interference costs at all during the acquisition of multiple handling techniques, and do experience small memory interference effects. This would be consistent with the model of flower handling described in Chapter 2 that relies on associative learning and not on acquisition of different motor patterns. Bumblebees are capable of learning multiple associations at the same time (Schencking, 1969), but they experience interference when required to switch repeatedly between them (Strang & Sherry, 2014). Thus, bumblebees here were successfully able to acquire multiple handling techniques without interference.

Repeated switching was required for Experiment 2, but again no evidence of interference costs was found. Bumblebees successfully improved on each of the tasks while acquiring them simultaneously and showed fluency and specificity in their use of the two required motor behaviours. The lack of interference on this task is consistent with findings from Chittka and Thomson (1997), where bumblebees that were required to switch frequently during training acquired both tasks more quickly than bees that were trained on a block design. The frequently used paradigm for testing interference is a block design (Lewis, 1986; Woodward & Laverty, 1992; Gegear & Laverty, 1995; Gegear & Laverty, 1998), with bees learning one task to

asymptotic performance and then being switched to the second task. This difference in design could be responsible for the complete absence of switching costs found here.

3.4.2 Flower Complexity

Previous investigations of costs of switching in bumblebees have found that the costs are more significant for complex flowers (Gegear & Laverty, 1995), and can even disappear when foraging on simple flowers (Gegear & Laverty, 1995). Consequently, it is necessary to consider the possible effect of complexity of flower morphology on the results here. Is it possible that the absence of interference costs is due to morphological simplicity of the model flowers? It is unlikely that this was the case. Simple flowers are defined as having nectaries that are exposed and both tasks described here required a component of the apparatus to be moved in order to access the sucrose reward. The flower used for the up task was intentionally designed to involve greater complexity than previously designed models, and the lengthy acquisition curves generated for that model and described in Chapter 2 support its morphological complexity. The down task did not undergo the same extensive development process as the up task. However it did require an entirely distinct motor pattern for success. It has been suggested that in some cases it is not the complexity of flower morphology that is important for interference costs, but rather the difference in morphology between two different flowers (Gegear & Laverty, 1995). The tasks here shared general properties, but the key morphological features related to successfully accessing the rewards were distinct.

3.4.3 Learning-to-learn

A surprising finding here was the ability of bees to learn general properties of the task and apply them to both variations, producing positive transfer of learning rather than interference. The general learning that occurred here was largely due to learning the location of

the reward. During initial trials bees spent significant time interacting with the exit door that could not be opened, but in later trials they focused their efforts exclusively on the door that could be opened and provided access to the sucrose reward. Learning-to-learn or positive transfer between tasks is widely known to occur in animals (Shettleworth, 1998; Shettleworth, 2009) and has been a particular topic of interest in research with object manipulation and tool use tasks (Povinelli, 2000; Martin-Ordas, Call, & Colmenares, 2008; Shettleworth, 2009). It has also been observed in flower handling work in the past (Laverty, 1994a). Even in light of previous research on learning-to-learn the extent to which positive transfer facilitates bees' performance on the tasks used here was still surprising. One factor that may have exaggerated bees' extraction of general information about flowers was the absence of any natural floral cues that would aid bees in locating nectar in real flowers. It widely known that flowers provide cues to the locations of their rewards through nectar guides and other cues (Leonard & Papaj, 2011). In the artificial flowers used here such cues were not present, so bees needed to learn the location of the rewards. This learning requirement, that may not present when foraging in the wild, could be responsible for the large learning-to-learn effect observed here.

3.4.4 Alternative mechanisms of flower constancy

With the interference hypothesis no longer considered to be a sufficient explanation for flower constancy, a perspective supported by the findings here, there needs to be an alternative explanation. As with most topics, when a dominant hypothesis is challenged multiple other hypotheses take its place. In the case of flower constancy these alternative hypotheses range from appeals to memory limitations (Raine & Chittka, 2007) to use of a search image while foraging that is susceptible to interference (Goulson, 2000; Gegear & Laverty, 2005), to

consideration of interacting effects all contributing to constancy (Chittka, Thomson, Waser, 1999).

Hypotheses regarding memory dynamics and limitations are consistent with the data here. My data suggest that although bees are not engaging in motor learning during flower handling, they are forming associations with each flower and those associations must be stored and accessed for bees to forage successfully. Raine and Chittka (2007) hypothesized that only information for one, or very few, flowers could be maintained in short-term memory while a bee was foraging. If the bees were to switch to foraging on a different species they would then have to retrieve information, and all previous associations, for that flower from long-term memory prior to making the switch. The intervals between flower visits were compared to switch likelihood for foraging bumblebees and the hypothesis was supported, in that bees were more likely to switch species following long intervals than following short intervals between flowers (Raine & Chittka, 2007). This supports the role of memory in flower constancy, just a different type of memory than originally suggested by Darwin.

3.4.5 Conclusions

Darwin's suggestion that bees are constant in the same way that an artificer executes one task at a time has all of the elegance and appeal of most of his ideas, but unfortunately does not seem to be the explanation for bumblebee constancy. The goal of this chapter was to determine why such a logically sound hypothesis was unsupported. It is clear from the data that when bees are required to switch between different flower morphologies they switch between two innate motor patterns and then associate the successful motor pattern with the particular flower. The bees here were able to switch between different handling techniques with little cost at the level of the motor behaviour. Bees do not suffer high costs of switching at the level of motor behaviours

required for flower handling because those behaviours are innate and not susceptible to interference. Interference effects observed are due to interference with associations of innate motor patterns and particular flowers, and not due to interference in motor patterns themselves.

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Chapter 4

4 Mushroom Body Volume and Reversal Learning

4.1 Introduction

Bumblebees show impressive flexibility in their generalist foraging strategy (Heinrich,1976; Heinrich, 1979/2004) and their ability to switch between flower handling techniques with only small efficiency costs (Woodward & Laverty, 1992; Raine & Chittka, 2007). How bees are able to generate such a large and flexible behavioural repertoire from a simple nervous system has received extensive consideration (Menzel & Giurfa, 1999). In the previous two chapters I addressed this problem indirectly by demonstrating that complex behavioural output such as flower handling can be generated through simple cognitive mechanisms. One strategy bumblebees have evolved to deal with limited neural capacity is to rely on simple cognitive solutions to ecological problems. However, the proposed mechanisms in Chapters 2 and 3 still require well-developed associative learning and neural structures that support those learning abilities. The mushroom bodies are considered to be the locus of learning in the bee brain (Strausfeld et al., 1998; Farris, 2005; Fahrbach, 2006), but their direct relation to measures of behavioural flexibility has not been thoroughly investigated. In this chapter, I explore the relation between the bumblebee mushroom bodies and behavioural flexibility, as measured by reversal learning.

4.1.1 Behavioural flexibility in bees

Behavioural flexibility can be broadly defined as an animal's ability to respond appropriately to changes in its environment (Coppens et al., 2010). This definition includes no information on how to operationalize behavioural flexibility, which has resulted in dramatically different ways of testing of flexibility in animals (Audet & Lefebvre, 2017). The many existing flexibility assays include quantifying foraging innovations (Sol et al., 2002), and performance on

problem solving (Auersperg et al., 2011; Benson-Amram & Holekamp, 2012) and switching tasks such as reversal learning (Bond et al., 2007; Strang & Sherry, 2014). In the frequently used reversal task an animal is trained on a discrimination, with one rewarding stimulus and one unrewarding stimulus, then the contingencies are reversed (Mackintosh, 1969; Bitterman, 1969; Davey, 1989; Shettleworth, 1998; 2010). The ability of an animal to recognize the change in reward contingencies and change their behaviour is considered a demonstration of flexibility. This measure can be used with any species that can learn to discriminate between stimuli, making it versatile, convenient, and popular for comparative and neuroscience studies.

There has been a good deal of research on reversal tasks in both honeybees (Menzel, 1969; Couvillon and Bitterman, 1986; Mota and Giurfa, 2010; Ben-Shahar et al., 2000) and bumblebees (Chittka, 1998; Strang & Sherry, 2014). The ability of bees to successfully perform a reversal has been found consistently but success on repeated reversals is more mixed (Couvillon and Bitterman, 1986; Mota and Giurfa, 2010; Chittka, 1998; Strang & Sherry, 2014). Bees appear to be successful at repeated reversals when given large numbers of trials and the opportunity to reach asymptotic performance or a similar criterion on both the initial and the reversed discrimination (Chittka, 1998; Strang & Sherry, 2014). Bees tend to respond randomly when neither of these two conditions is met (Couvillon and Bitterman, 1986; Mota and Giurfa, 2010). Despite the differences in these studies of repeated reversal learning, the pattern of learning and errors by bees in initial discrimination acquisition and a single reversal is largely consistent. Bees generate learning curves for the initial discrimination, make repeated choices to the no longer rewarded stimulus following the reversal (perseverative errors), and then acquire the reversed discrimination. This pattern is typical of animals that use associative learning to solve reversal tasks (Mackintosh, 1969; Bitterman, 1969; Davey, 1989; Shettleworth, 1998;

2010), suggesting that bees are employing associative learning to behave flexibly in reversal tasks. Additional evidence for an associative mechanism for bees' flexibility comes from detailed examination of the pattern of errors and correct choices on the serial reversal task (Strang & Sherry, 2014). When bumblebees are required to make repeated reversals, the efficiency with which they switch improves, though the pattern of errors that they make suggests that this improvement is actually due to proactive interference rather than more complex rule learning (Strang & Sherry, 2014).

Studies of reversal learning show that behavioural flexibility by bees on these tasks might be due to a memory limitation and consequent failure of associative learning (Strang & Sherry, 2014). It follows from this that there should be an inverse relation between performance on discrimination learning tasks and reversal tasks. Bees that are exceptional learners should perform poorly on reversal tasks, and those that have difficultly acquiring associations should be more susceptible to interference effects and more easily reverse the associations that they have formed. Raine & Chittka (2012) found no such learning vs. flexibility trade-off, however, when they measured discrimination acquisition and reversal learning in bumblebees. Bumblebees (*Bombus terrestris*) learned to associate yellow artificial flowers with sucrose reward, and then were exposed to a reversal where blue flowers provided high value sucrose reward. Bees that acquired the initial discrimination quickly also reversed the discrimination quickly (Raine & Chittka, 2012). This finding argues against the hypothesis that behavioural flexibility in bees is due in part to limitations in associative learning. Additionally, this result is relevant to questions about the neural correlates of reversal learning. A study of the development of mushroom bodies in foraging honeybees found that increases in volume of the calyces was due to increases in dendritic branching (Farris, Robinson, and Fahrbach, 2001). The increased volume and

branching is proposed to support the increased need to form associations and learn during foraging (Farris et al., 2001), suggesting that bees with larger mushroom bodies have a greater capacity to form and maintain associations compared to bees with smaller mushroom bodies. If flexibility is due to a failure of memory then reversal should have a negative correlation with brain regions that support strong associative learning, such as the mushroom bodies. In contrast, if reversal learning is supported by similar cognitive processes as associative learning, as proposed by Raine & Chittka (2012), then one would expect both discrimination learning and reversal learning to have a positive correlation with brain regions that support learning and memory.

Reversal learning is an ideal candidate to investigate the relation between the bumblebee brain and behavioural flexibility because of the extensive investigation of reversal learning in bees and the thorough characterization of bee behaviour on the task.

4.1.2 Learning and the mushroom bodies

From their initial discovery by Dujardin (1850) the mushroom bodies of the insect brain have been linked to learning and memory because of their size and prominence in the insect brain. Since Dujardin first proposed a relation between the mushroom bodies and learning that relation has been experimentally demonstrated (De Belle & Heisenberg, 1994; Komischke et al., 2005; Gronenberg & Couvillon, 2010). De Belle and Heisenberg (1994) removed the mushroom bodies of Drosophila through chemical ablation and observed severe impairments in associative learning in experimental flies, despite otherwise normal activity levels and mating success. Komischke et al. (2005) used chemical ablation to study mushroom bodies and learning in honeybees and performed unilateral rather than bilateral ablations. This technique allowed comparison of learning with and without intact mushroom bodies in the same individual by

conducting olfactory learning trials using stimuli applied to either the ablated side or the intact side of the bee. Honeybees showed intact olfactory conditioning when the intact hemisphere was employed, but showed impairment when they were trained on their ablated side (Komischke et al., 2005).

There is considerable diversity in mushroom body size and morphology in insects (Strausfeld et al., 1998), and it has been suggested that the volume of the mushroom bodies is related to learning and memory capacity (Strausfeld et al., 1998; Farris, 2005; Fahrbach, 2006). *Hymenoptera* (bees, ants, and wasps) have larger mushroom bodies than other insects (Fahrbach, 2006). This investment in larger mushroom bodies by *Hymenoptera* could be due to increased social interaction (O'Donnell et al., 2011) or increased foraging demands (Mares et al., 2005). Additionally, mushroom body size increases at the onset of foraging in bumblebees (Riveros & Gronenberg, 2010), honeybees (Withers et al., 1993; Withers et al., 1995) and other *Hymenoptera* (O'Donnell et al., 2004; Withers et al., 2007), a transition during which enhanced associative learning abilities may be advantageous.

Aside from ablation studies, much of the evidence for a relation between mushroom body volume and learning relies on the assumption that increased social or foraging demands are paired with increased cognitive demands. Gronenberg and Couvillon (2010) directly explored the relation between mushroom body volume and learning in bees. Gronenberg and Couvillon (2010) trained honeybees on an olfactory discrimination task and compared performance to mushroom body volume. A positive correlation was found between total brain volume and learning, and between mushroom body volume and learning (Gronenberg & Couvillon, 2010).

Given the previously observed relation between mushroom body volume and associative learning (Gronenberg & Couvillon, 2010), and the associative mechanisms involved in

behavioural flexibility as measured by reversal learning (Shettleworth, 1998/ 2010), mushroom body volume is a suitable brain measure for exploring the neural correlates of flexibility in bumblebees.

4.1.3 Goal of this chapter

The purpose of this chapter is to explore the neural structures that support behavioural flexibility in bumblebees measured by reversal learning. Bees were trained on a colour discrimination reversal and their performance was compared to the volume of the mushroom bodies and subdivisions within the mushroom bodies. Previous work supports the hypothesis that a positive relation exists between mushroom body volume and initial discrimination learning, but the relation between mushroom body volume and reversal learning is less clearly predicted. If successful reversal learning is due to interference with a previously formed association then a negative correlation between mushroom body volume and reversal performance would be predicted. If successful reversal learning is the product of the same learning mechanism that is responsible for initial discrimination learning as proposed by Raine & Chittka (2012), then a positive correlation between mushroom body volume and both discrimination and reversal learning would be predicted.

4.2 Methods

4.2.1 Subjects and housing

Subjects were 16 bumblebee workers (*Bombus impatiens*) from 2 colonies obtained from Biobest Canada Ltd. (Leamington, ON). Bee colony boxes, provided by Biobest Canada Ltd, were placed in a 3.0 X 8.5 m room (Figure 4-1) with a 12h light/dark cycle, light onset 7am. Bees had access to the housing room through a 2cm wide exit in the colony box that was open 24h/day. Bees had access to sucrose solution in six foraging patches consisting of white 30.5 X

30.5 cm Smoothfoam™ polystyrene sheets that were placed on four tables in the housing room. Each foraging patch contained five artificial flowers that consisted of 7ml plastic microtubes (Axygen®,Union City, CA) with the caps removed and a 5cm wide clear plastic corolla around the microtube. Each of the artificial flowers was provisioned with 15-17% sucrose solution that was replenished as needed, providing *ad libitum* sucrose for the bee colony. Pollen was given daily to the colony through a small opening in the lid of the colony container. Colonies were given at least five days after arrival to begin foraging and habituate to their housing conditions before bees were tested.

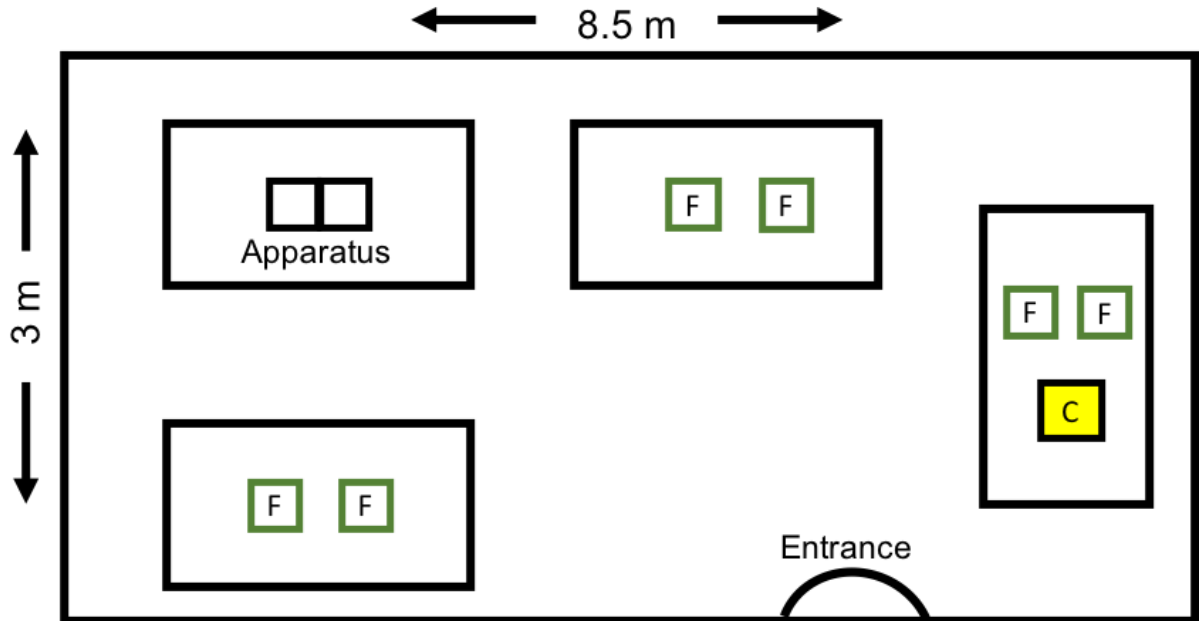

Figure 4-1 Housing room. The bee colonies were contained in colony boxes (C) and given unrestricted access to the housing room. The bees collected sucrose solution from six foraging patches (F) placed on tables in the room. All testing occurred at the table farthest from the colony where the apparatus was placed for the entire duration of the experiment.

Prior to testing, bees were collected while foraging, restrained in a marking tube, and tagged for individual identification with either plastic number tags (Betterbee Inc., Greenwich, NY) affixed with cyanoacrylate glue or Posca paint markers (Mitsubishi Pencil Co.). Bees were identified during testing by their colour and number.

4.2.2 Apparatus

The apparatus (Figure 4-2) consisted of two 20.3 X 20.3 X 12.7cm boxes constructed from Elmer's® 1/2" foam board with a clear plastic lid that covered the entire width and 12.7cm of the depth of the top of each box. On the entrance side of each box the top front panel was partially removed, lowering the height of the box to 7.6cm, to allow bees to enter the apparatus. Each box had additional pieces of foam board attached to the left and right sides of the box to support the test stimuli. The stimuli consisted of 20.3 X 20.3 cm blue and yellow Creatology™ foam sheets (Michaels Stores Inc.) which were inserted into the box to create a platform on which the bee could land. Underneath the stimuli there were two artificial flowers, one that contained a reward and one that did not. The flowers were attached to a sliding piece of polystyrene that could be manipulated from outside the apparatus to make either the empty or full artificial flower accessible through a hole in the stimuli. This setup allowed bees to make their choice between the stimuli in the absence of reward, but to be immediately rewarded upon making a correct choice. Following each testing session, the colour stimuli were removed from the apparatus, white sheets of Creatology™ foam were inserted into the apparatus, and the artificial flowers were arranged such that the rewarding flowers were available. The colour stimuli were wiped with 70% isopropyl alcohol after all testing sessions to remove any odours left by bees during testing.

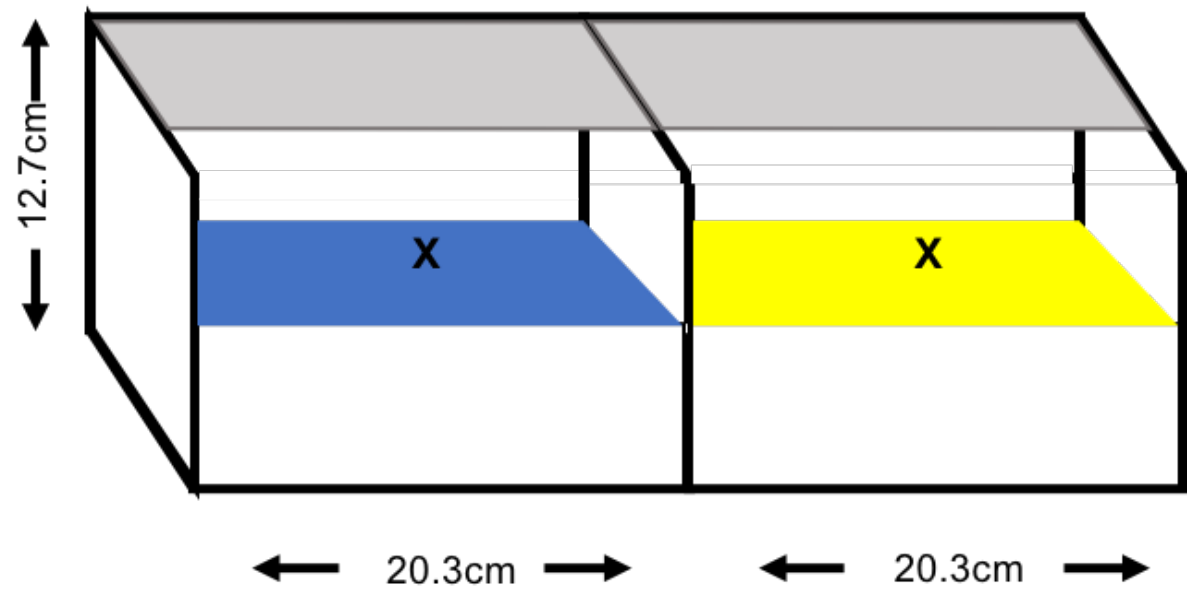

Figure 4-2 Apparatus. The apparatus consisted of two boxes with clear lids that partially covered the top of the boxes. Coloured stimuli (blue and yellow) were placed in the boxes during testing and formed the floor of each box. The 'X's indicate the location in which a sucrose reward was accessible on correct trials and an empty artificial flower was encountered on incorrect trials.

4.2.3 Habituation

The testing apparatus was available to bees in the housing room for at least 24h prior to testing and then continued to be available throughout the experiment when testing was not occurring. During habituation the apparatus was baited with 40% sucrose solution.

4.2.4 Testing Procedure

In each session bees completed 30 trials. The first 10 trials consisted of a discrimination between blue and yellow. The initially rewarded colour (S+) was counterbalanced across bees. The final 20 trials were a reversal of the initial discrimination. In each trial the apparatus contained both colour stimuli, blue and yellow, with one stimulus in each box. The left/right position of the colours was pseudorandomized across trials. Each colour appeared an equal number of times in the left and right positions within a ten trial block, and no colour remained in the same position for more than three trials in a row.

Entrance into the apparatus past the point at which it was lidded was considered a choice. Bees were allowed to make multiple choices in a single trial, but only the first choice was used in analysis. When a bee made a correct choice the filled rewarding artificial flower filled with 40% sucrose solution was positioned under the opening in the floor of the apparatus. When a bee made an incorrect choice they encountered an empty artificial flower. A trial was completed when the bee left the area of the apparatus. When the bee returned to the area of the apparatus choices were recorded as a new trial.

4.2.5 Histology

Bees were collected immediately following testing, cold anesthetized, and decapitated. Brains were removed from the head capsule in bee saline, a mixture of salts (NaCl, KCl, $CaCl_2$, $MgCl_2$), sugars (dextrose, fructose, sucrose), and distilled water mixed to match the osmolarity of bee hemolymph (Gronenberg, personal communication, 2010). Brains were then immediately placed in alcoholic Bouin's fixative and stored overnight. Brains were dehydrated in ethanols, cleared in xylene, and embedded in Paraplast®.

Brains were sliced on a microtome in 5μm coronal sections. Brain sections were deparaffinized and stained with Cason's trichrome stain (Cason, 1950). Area measurements were taken from every fifth section resulting in a section interval of 20μm. Area measurements were obtained from digital images captured using a Leica DM5500 B microscope by tracing structure outlines using ImageJ (Schneider, Rasband, & Eliceiri, 2012). Images used for quantifying total mushroom body volume, peduncle volume, and lobe volume were taken using a 5X objective. Images used to quantify the mushroom body subcompartments (lip, collar, basal ring) were taken using a 10X objective. Volume was calculated from the area measurement using the formula for the volume of a truncated cone.

Tissue loss during processing resulted in exclusion of four brains from analyses. Tissue loss that was restricted to one hemisphere occurred in four additional brains. In these brains the values from the intact hemisphere were used for both hemispheres.

4.2.6 Data Analysis

Analyses were done on the behavioural data, the relations between the behavioural data and the histology data, and the histology data alone, the latter to determine the relations among structures within the mushroom bodies. All analyses were done in RStudio®.

4.3 Results

All 16 bees completed the behavioural trials and are included in analysis of the behavioural data, but four bees are excluded from the histology.

4.3.1 Behavioural results

Bee choices for the behavioural task were separated into three different trial blocks, each with ten trials. The first trial block consisted of the discrimination trials, and blocks two and three consisted of the 20 reversal trials separated into two trial blocks.

Choice data were analyzed with a repeated measures ANOVA. Choices changed across trial blocks ($F(26, 2) = 42.39$, $p < .001$) (Figure 4-4). Tukey HSD pairwise comparisons showed that each trial block differed from each other trial block. The number of correct choices was higher in the discrimination than in the reversal trial block 1 ($p < .001$) or the reversal trial block 2 ($p = .01$). Performance on the two reversal blocks also differed from each other ($p < .001$), with bees making more correct choices in the second trial block than in the first.

4.3.2 Histology and behaviour correlations

Six volume measurements were calculated (total mushroom body volume, peduncle and lobes, calyx, collar, lip, and basal ring). The mean values for each of the volume measurements

are given in Table 4-1. Pearson's product moment correlation was used to relate volume measurements to discrimination learning, the 20 trials of reversal learning, and reversal learning separated into trial block 1 and trial block 2.

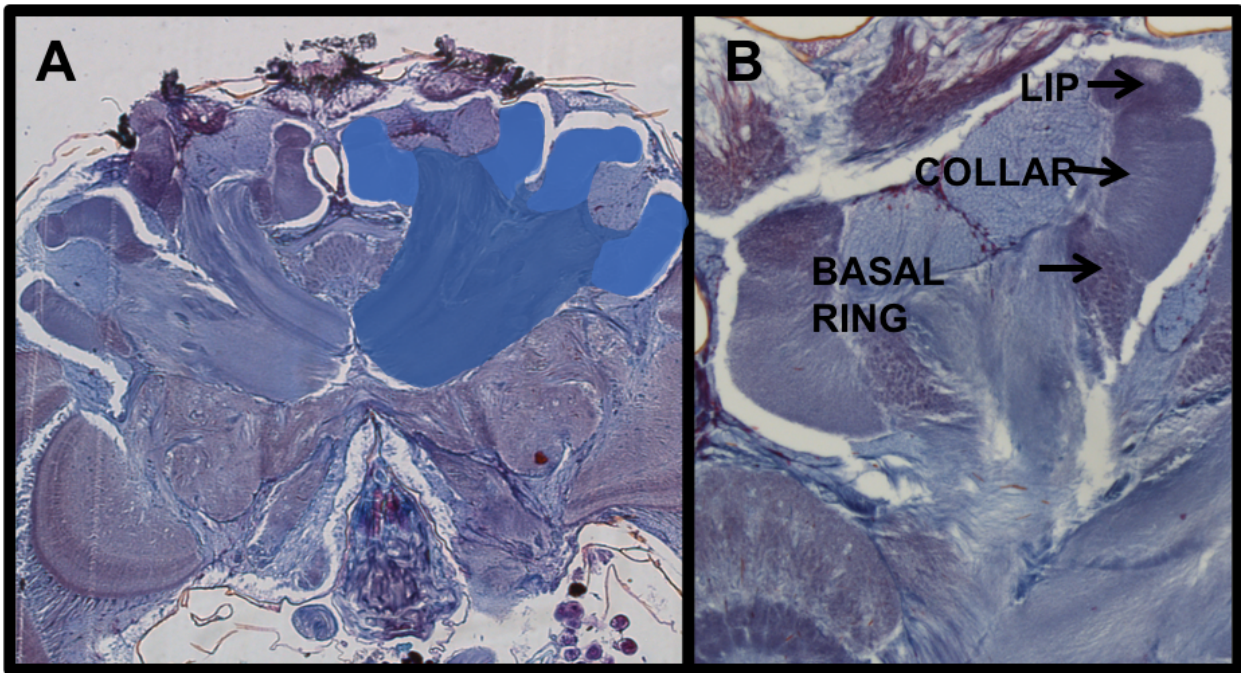

Figure 4-3 Coronal section of the bee brain. Panel A shows a coronal section stained with Cason's trichrome stain taken using 5x lens. The images shows both hemispheres of the bee brain including the left and right mushroom bodies, each of which consists of medial and lateral calyces, shaded in light blue, and the peduncle and lobes, shaded in dark blue. Panel B shows a mushroom body calyx at 10x objective lens. Staining with Cason's trichrome stain results in the subcompartments of the calyx (collar, lip, basal ring) being visible and with easily discernable boundaries.

There were no significant correlations between volume measurements and any of the behaviour measures.

Total mushroom body volume did not significantly correlate with performance on the discrimination trial block ($r(10) = .48$, $p = .11$), overall reversal performance ($r(10) = .1$, $p = .75$), reversal trial block 1 ($r(10) = -.003$, $p = .99$), or reversal trial block 2 ($r(10) = .23$, $p = .5$) (Figure 4-4).

Table 4- 1 Mean mushroom body volume measurements

Brain Region	Mean Volume (mm^3)	Standard Deviation (mm^3)
Total Mushroom Body	0.195	0.022
Calyx	0.123	0.012
Collar	0.089	0.008
Lip	0.021	0.003
Basal Ring	0.013	0.001
Peduncle + Lobes	0.072	0.014

There were no significant correlations between combined peduncle and lobe volume and discrimination learning ($r(10)$ = .4, p = .19), overall reversal performance ($r(10)$ = -.07, p = .82), reversal trial block 1 ($r(10)$ = -.122, p = .7), or reversal trial block 2 ($r(10)$ = .02, p = .95) (Figure 4-5).

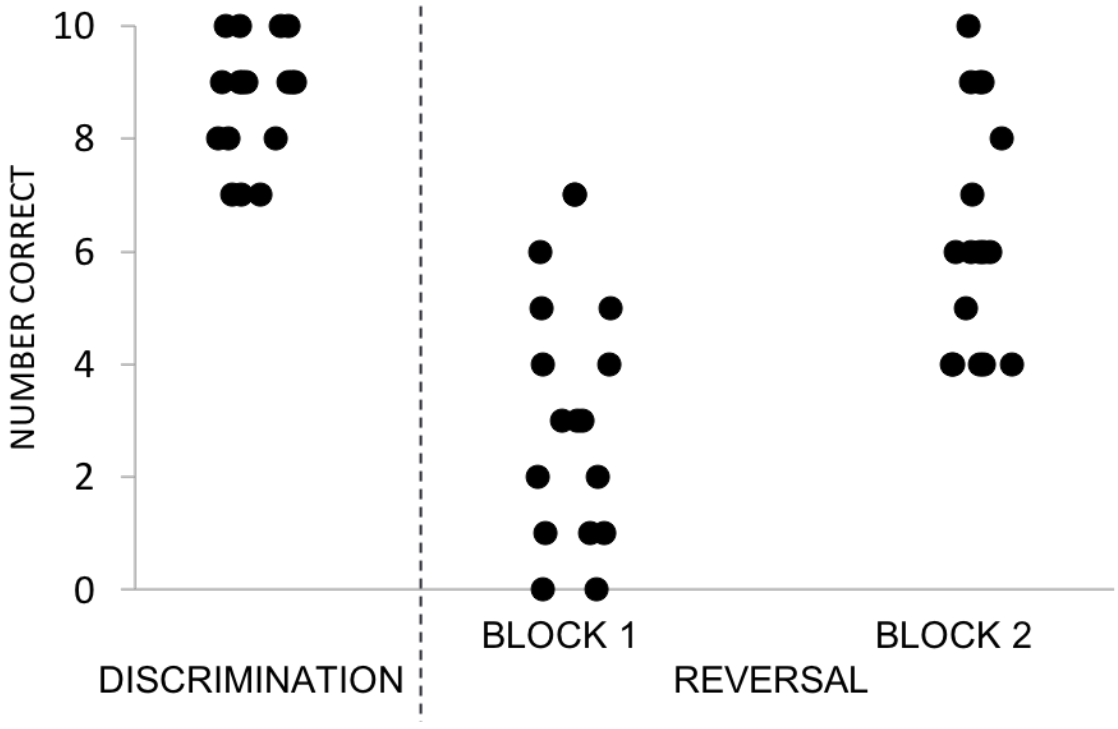

Figure 4-4 Discrimination and reversal learning. Discrimination learning data is shown as number correct out of ten trials on the left side of the dashed line. Reversal learning data is

shown or the right side of the dashed line in two separate trial blocks, each consisting of ten trials. Bees showed a typical reversal learning pattern of easy acquisition of the initial discrimination, significant perseverative errors after the reversal (Block 1), and then acquisition of the reversed discrimination (block 2). The horizontal distribution of points for each block of trials was created artificially to make the individual data points in each category visible.

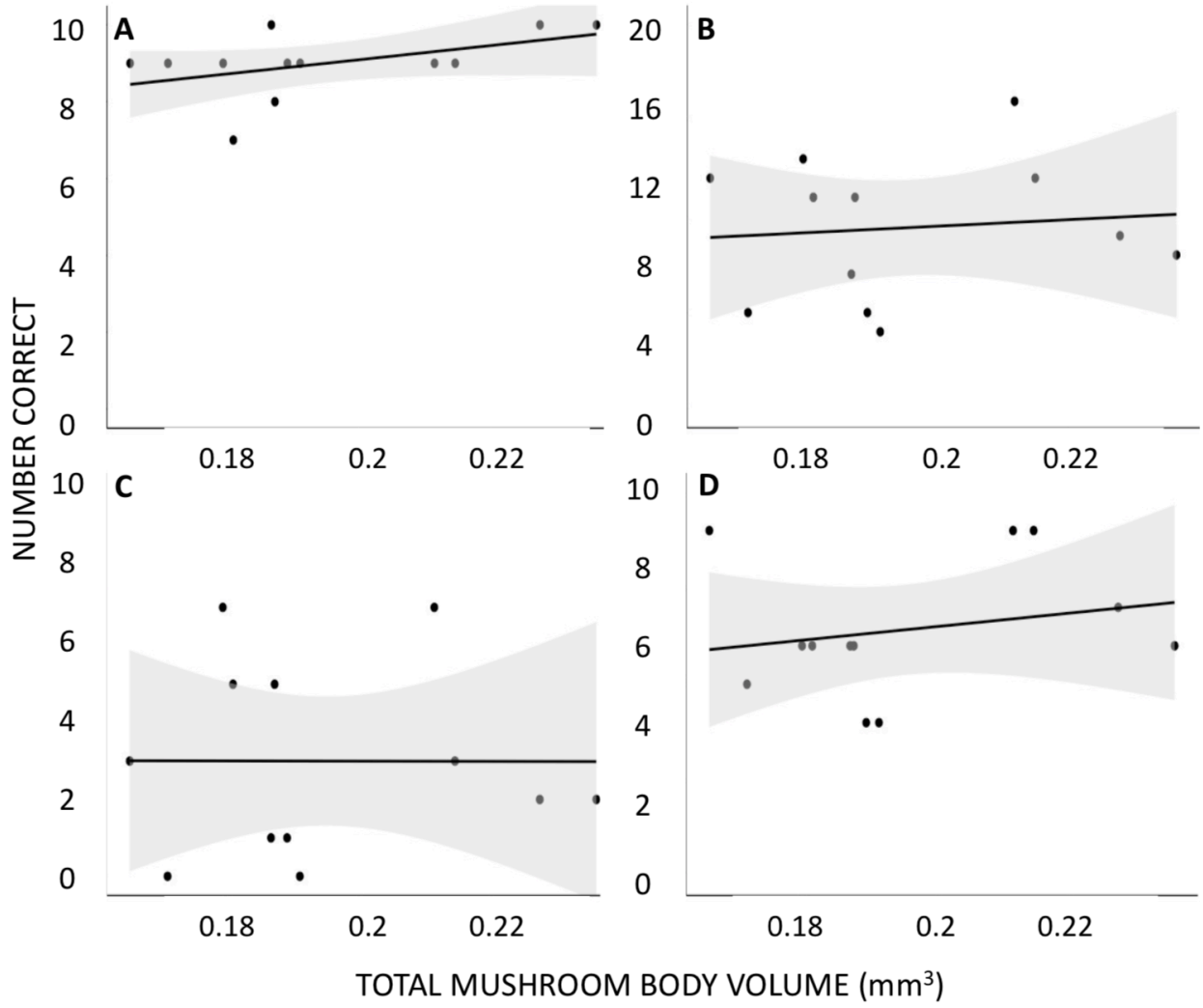

Figure 4-5 Total mushroom body volume and learning. Panel A shows the non-significant correlation between total mushroom body volume and the number of correct choices on the discrimination. Panel B shows the non-significant correlation between total mushroom body volume and the number of correct choices on the reversal. Panels C and D show non-significant correlations between total mushroom body volume and number correct on the first and second block of reversal trials respectively. The gray shading in each panel shows the 95% confidence interval for the correlations.

Calyx volume did not significantly correlate with discrimination learning ($r(10) = .43$, $p = .16$), reversal learning ($r(10) = .28$, $p = .38$), reversal block 1 ($r(10) = .14$, $p = .66$), or reversal

block 2 (*r(10)* = .39, *p* = .21) (Figure 4-6). The collar subcompartment did not significantly correlate with the discrimination (*r(10)* = .4, *p* = .19), overall reversal (*r(10)* = .29, *p* = .36), reversal block 1 (*r(10)* = .13, *p* = .69), or reversal block 2 (*r(10)* = .42, *p* = .18) (Figure 4-7). Lip volume did not significantly correlate with the discrimination (*r(10)* = .5, *p* = .86), overall reversal (*r(10)* = .05, *p* = .86), reversal block 1 (*r(10)* = -.04, *p* = .9), or reversal block 2 (*r(10)* = .18, *p* = .59) (Figure 4-8). Finally, the basal ring did not significantly correlate with discrimination learning (r(10) = .09, p = .78), overall reversal (r(10) = .44, p = .15), reversal block 1 (*r(10)* = .45, *p* = .14), or reversal block 2 (*r(10)* = .28, *p* = .37) (Figure 4-9).

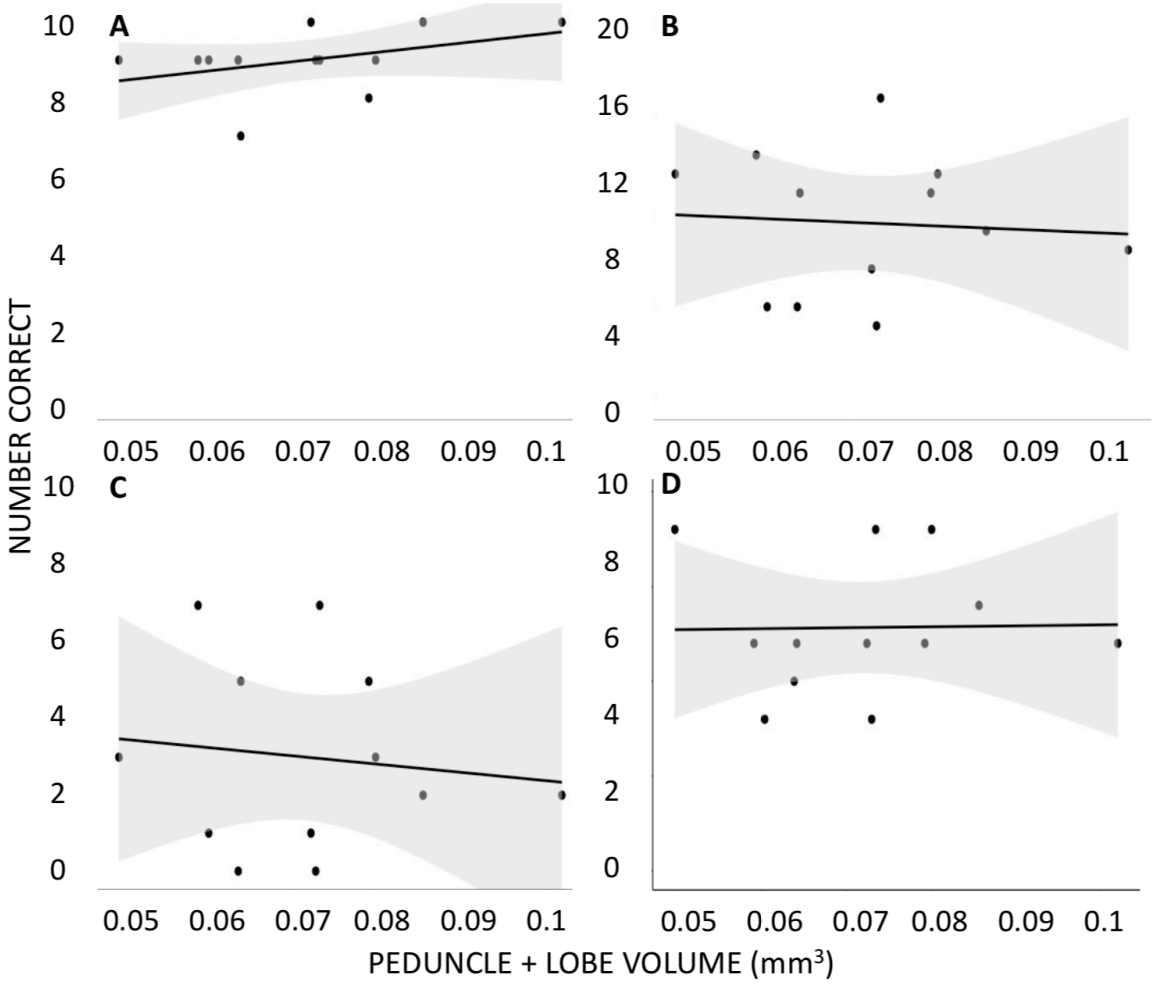

Figure 4-6 Combined peduncle & lobe volume and learning. The four panels show non-significant correlations between combined peduncle and lobe volume and discrimination

learning (Panel A), overall reversal learning (Panel B), block 1 of reversal learning (Panel C) and block 2 of reversal learning (Panel D).

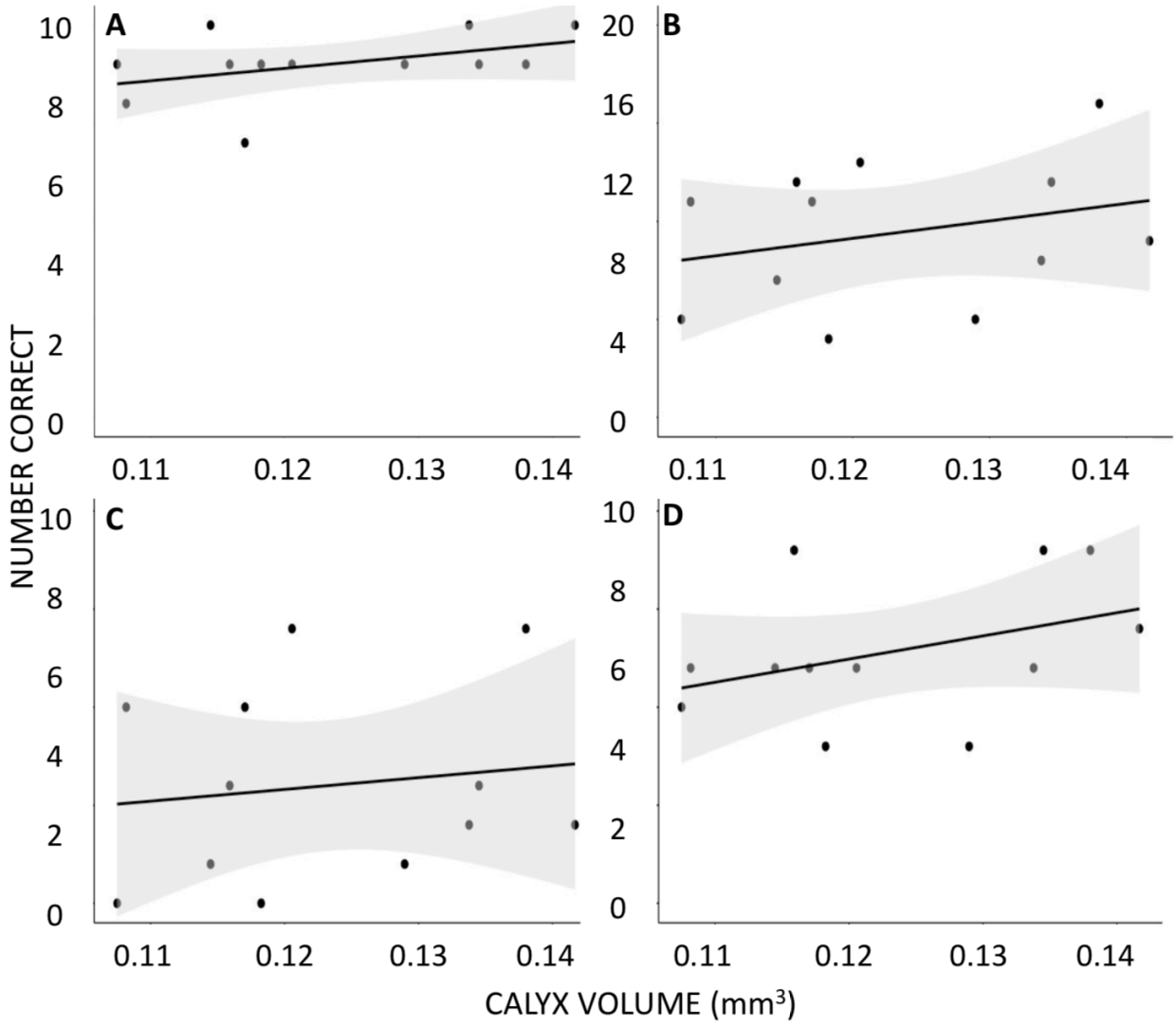

Figure 4-7 Calyx volume and learning. All four panels depict non-significant correlations between calyx volume and discrimination learning (Panel A), overall reversal learning (Panel B), reversal block 1 (Panel C), and reversal block 2 (Panel D).

4.3.3 Mushroom body volume correlations

The contributions of the internal mushroom body structures to the total mushroom body volume are shown in Figure 4-10. The combined peduncle and lobes accounted for 36.84% of the total mushroom body volume. The calyces accounted for the remaining 63.16%. The collar region of the calyces was the largest calyx subcompartment and the largest contributor to total

mushroom body volume at 45.62%. The lip subcompartment was the second largest part of the calyces at 10.6%, and the basal ring was the smallest subcompartment at 6.94% to total mushroom body volume.

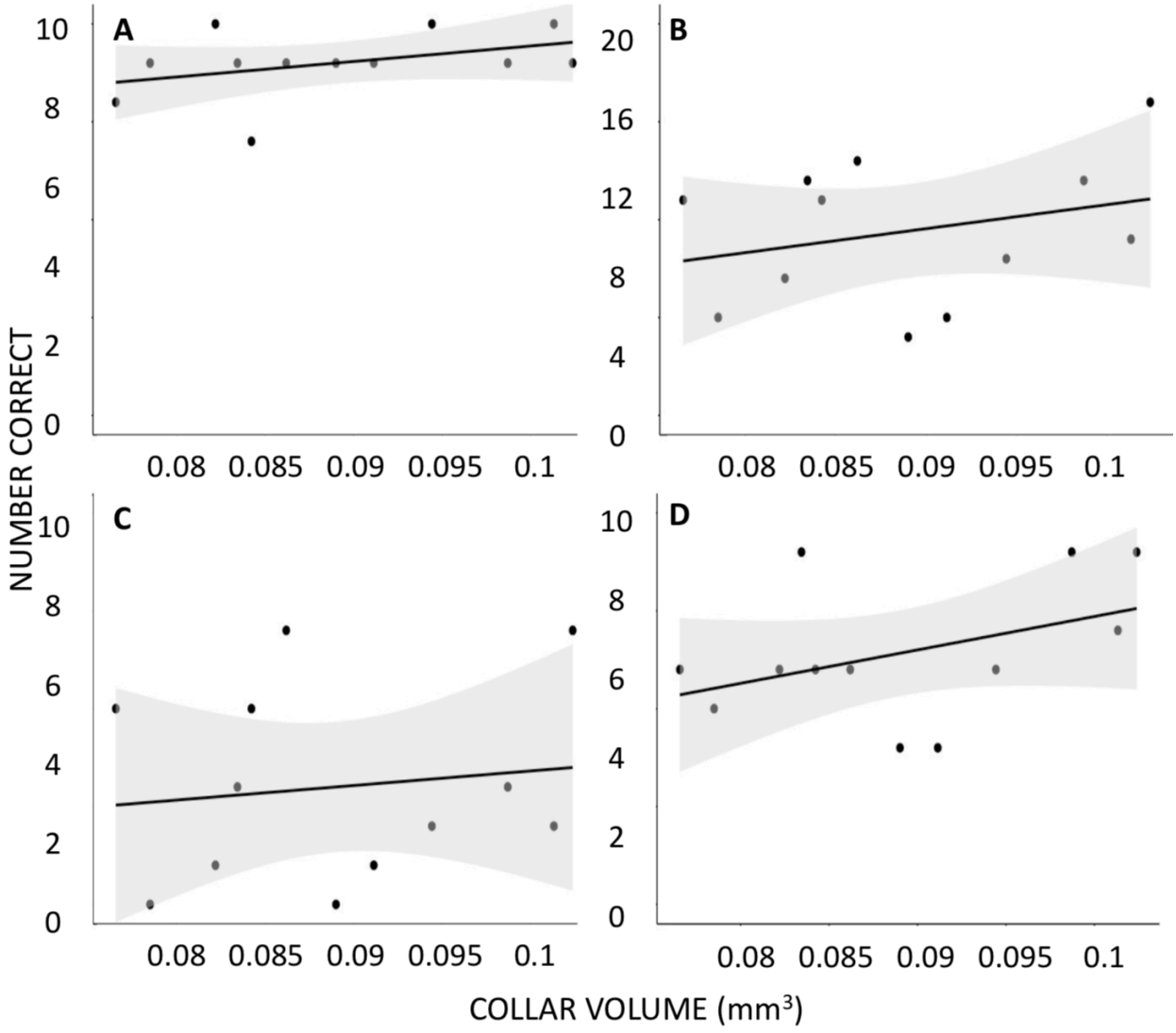

Figure 4-8 Collar volume and learning. Non-significant correlations are shown between collar volume and discrimination learning (Panel A), overall reversal learning (Panel B), reversal block 1 (Panel C), and reversal block 2 (Panel D).

The correlations for total mushroom body volume with all internal mushroom body structures are shown in Figure 4-11. Total mushroom body volume significantly correlated with volumes of

the peduncle and lobes ($r(10) = .89$, $p < .001$), calyx ($r(10) = .83$, $p < .001$), collar ($r(10) = .8$, $p = .002$), lip ($r(10) = .64$, $p = .03$), and the basal ring ($r(10) = .71$, $p = .01$).

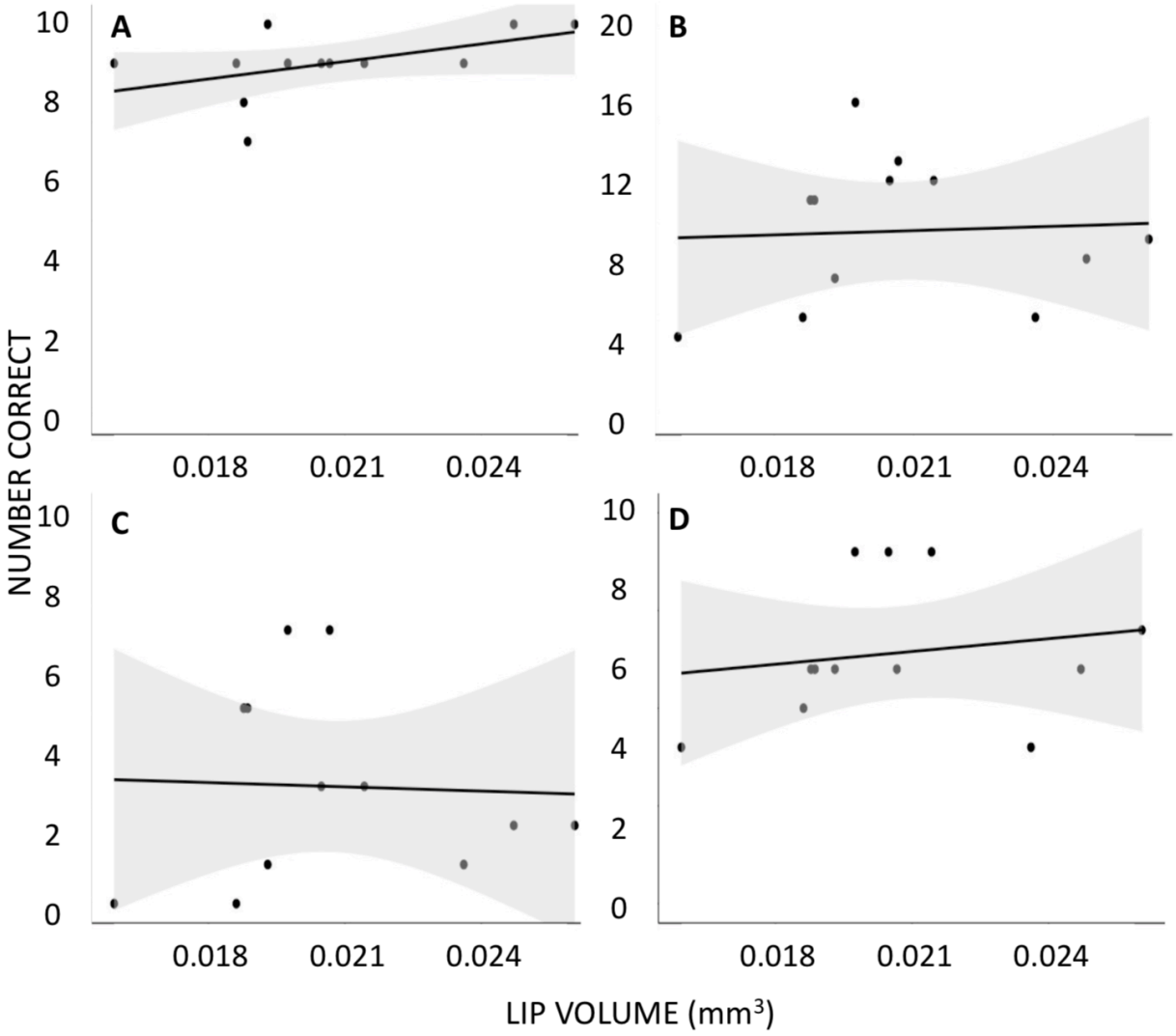

Figure 4-9 Lip volume and learning. Non-significant correlations between lip volume and discrimination learning (Panel A), overall reversal learning (Panel B), reversal learning block 1 (Panel C), and reversal learning block 2 (Panel D) are shown.

The correlations for calyx volume are shown in Figure 4-12. Total calyx volume was significantly correlated with all of the subcompartments, the collar ($r(10) = .98$, $p < .001$), the lip ($r(10) = .71$, $p = .01$), and the basal ring ($r(10) = .82$, $p = .001$). Total calyx volume did not significantly correlate with combined peduncle and lobe volumes ($r(10) = .49$, $p = .11$). Within

the calyces, the collar was not significantly correlated with the lip ($r(10) = .56$, $p = .06$), but the collar was significantly correlated with the basal ring ($r(10) = .8$, $p = .002$) (Figure 4-13). The lip and the basal ring were not significantly correlated ($r(10) = .41$, $p = .19$) (Figure 4-13).

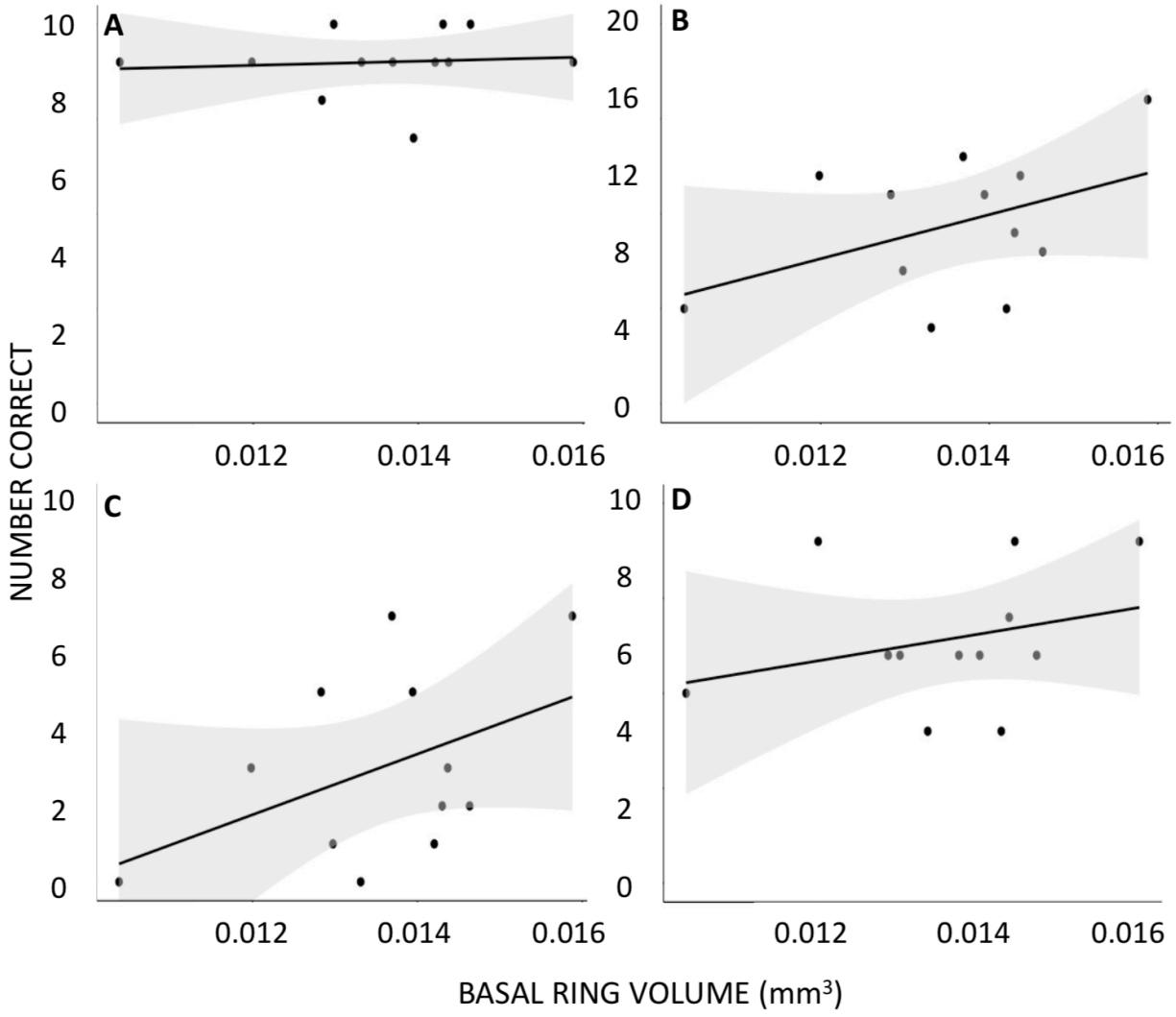

Figure 4-10 Basal ring volume and learning. Correlations between basal ring volume and discrimination learning (Panel A), overall reversal learning (Panel B), reversal block 1 (Panel C), and reversal block 2 (Panel D) were all non-significant.

Combined peduncle and lobes volume did not significantly correlate with any of the mushroom body subcompartments; the collar ($r(10) = .45$, $p = .15$), the lip ($r(10) = .41$, $p = .18$), or the basal ring ($r(10) = .44$, $p = .15$) (Figure 4-14).

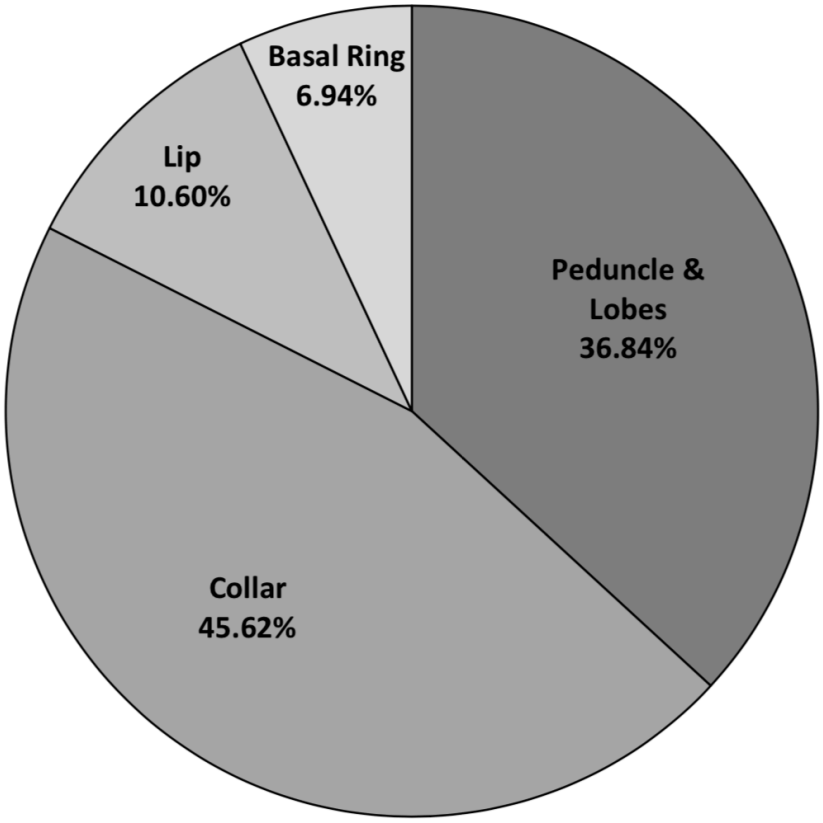

Figure 4-11 Mushroom body composition. The mushroom bodies can be divided into the calyces, peduncle, and lobes. Here the volume of the peduncle and lobes are combined and volume of the calyces is separated into the subcompartments (collar, lip and basal ring). The collar subcompartment of the calyces was the largest contributor to mushroom body volume.

4.4 Discussion

Previous investigations of learning in the bee brain have found a relation between mushroom body volume and learning abilities (Gronenberg & Couvillon, 2010) but that relation was not found here. Additionally, no relation was found between behavioural flexibility, measured by reversal learning, and mushroom body volume. The importance of the mushroom bodies to learning in insects is supported by previous research (De Belle & Heisenberg, 1994; Komischke et al., 2005; Gronenberg & Couvillon, 2010), making it unlikely that the results here

indicate the absence of any relation between learning and the mushroom bodies or reversal learning and the mushroom bodies. It is more likely that the relation between the mushroom bodies and learning was not observable at the level of analysis of mushroom body volume in this study, despite observations of a relation at this level of analysis previously (Gronenberg & Couvillon, 2010).

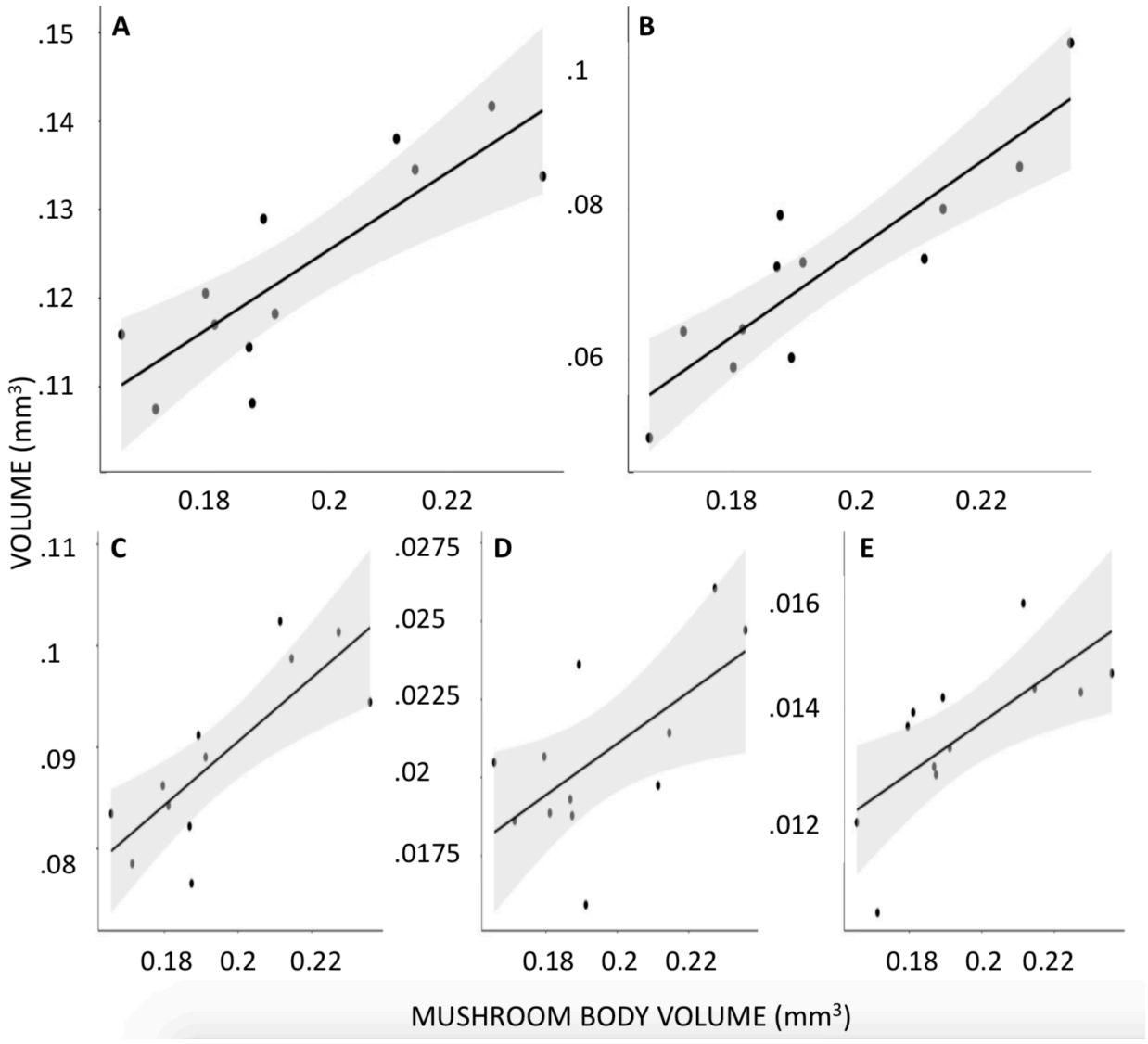

Figure 4-12 Total mushroom body correlations with internal mushroom body structures. The panels show total mushroom body correlations with calyx volume (Panel A), peduncle and lobes volume (Panel B), collar volume (Panel C), lip volume (Panel D), and basal ring volume (Panel C). All correlations were significant.

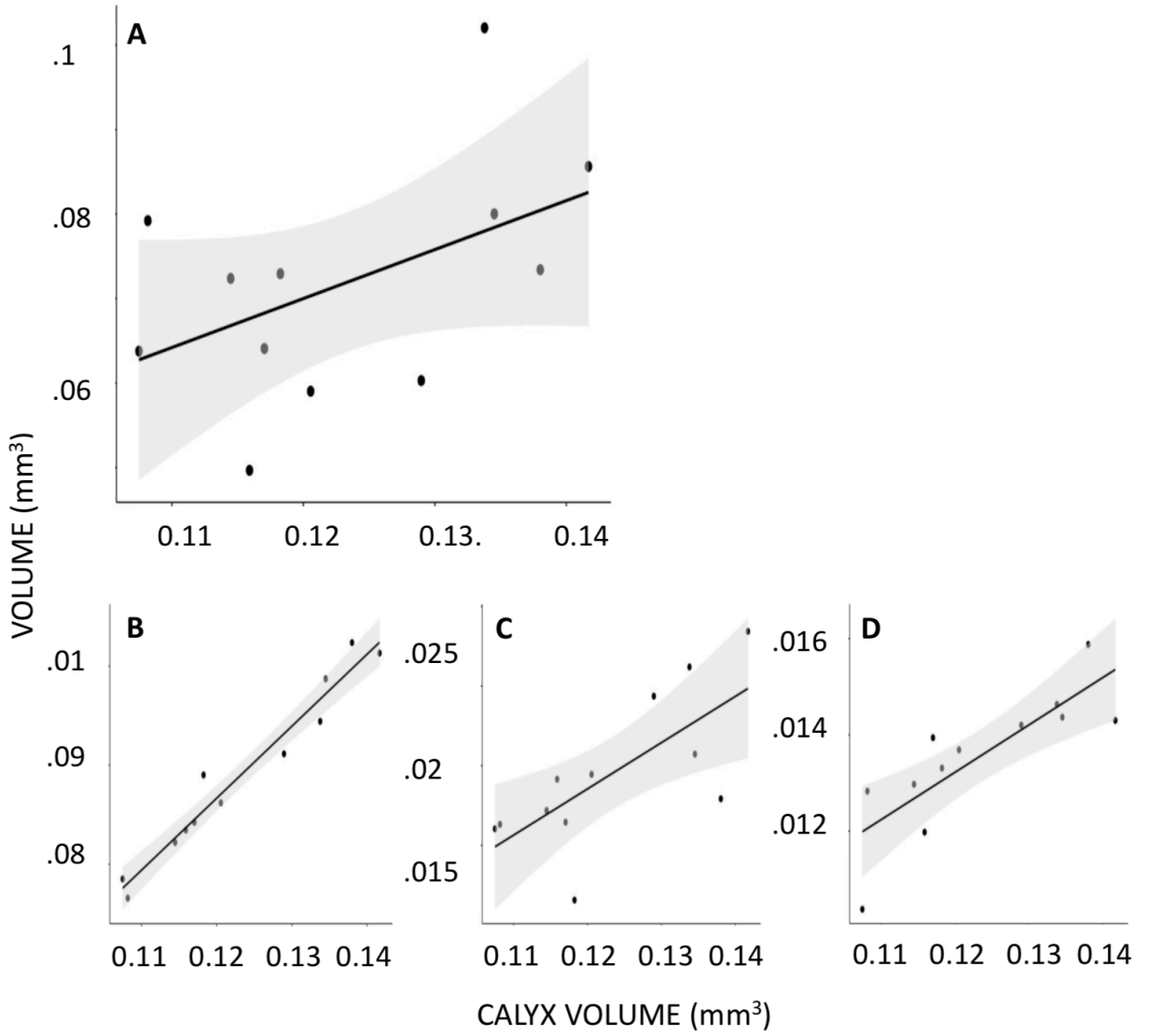

Figure 4-13 Calyx volume correlations. The nonsignificant correlation between total calyx volume and combined peduncle and lobe volume is shown in Panel A. The significant correlations between calyx volume and the calyx subcompartments are shown in bottom three panels (Panel B = collar volume, Panel C = lip volume, Panel D = basal ring volume).

4.4.1 Learning and the mushroom bodies

Bumblebees here showed the expected pattern of performance in the reversal learning task. They readily acquired the initial colour discrimination, made repeated errors when the reward contingencies were reversed, and then acquired the reversed discrimination. Bees did not acquire the reversed discrimination within twenty trials to the near error free degree that they acquired the initial discrimination, though improvement from the first block of reversal trials to

the second block suggests that they would have reached near perfect performance if given more trials. In successfully completing the discrimination and reversal, bees demonstrated learning and behavioural flexibility as expected, and provided a behavioural measure to relate to the mushroom body anatomical data.

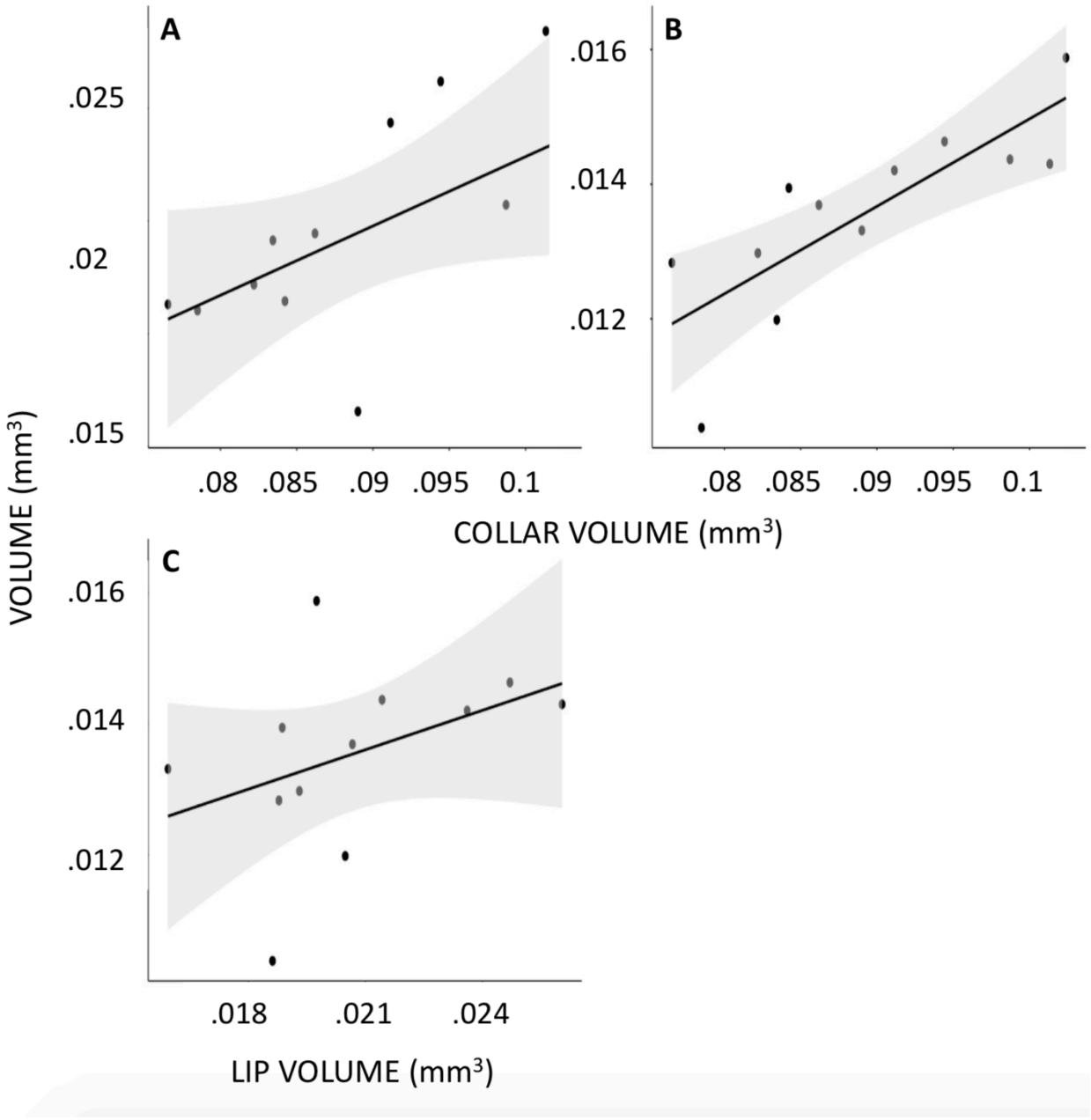

Figure 4-14 Mushroom body subcompartments correlations. Within the calyces, collar volume was significantly correlated with basal ring volume (Panel B), but not with lip

volume (Panel A). Lip volume and basal ring volume were not significantly correlated (Panel C).

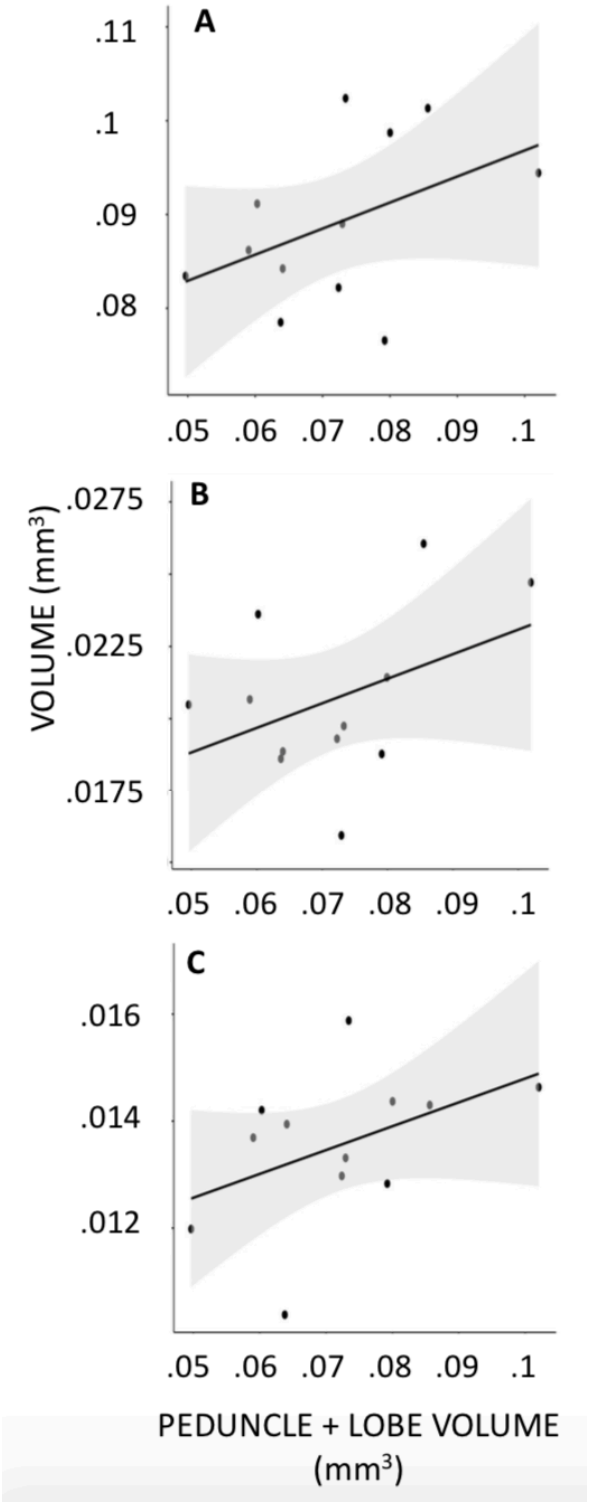

Figure 4-15 Combined peduncle and lobe correlations. Combined peduncle and lobe volume did not significantly correlate with the collar subcompartment of the calyces (Panel A), the lip (Panel B), or the basal ring (Panel C).

Correlations between bumblebee mushroom bodies and behaviour showed no significant relation between mushroom body volume as a whole or volumes of any of the component parts and behavioural learning and flexibility. This was surprising given the previous observation in honeybees of a relation between mushroom body volume and learning on a discrimination task (Gronenberg & Couvillon, 2010). There are a couple of possible explanations for the absence of a relation in this study. In Gronenberg and Couvillon's (2010) experiment honeybees were trained on an olfactory discrimination using the proboscis extension reflex procedure (PER), in which a bee is harnessed and extension of the proboscis is conditioned through application of an odour to the antennae paired with a sucrose reward (Bitterman et al., 1983). This procedure differs considerably from the procedure here in which bees were free-flying and completing a colour discrimination. One would expect considerable consistency in the neural structures responsible for associative learning regardless of the sensory domain. Projections from sensory structures are segregated, however, in the mushroom body clayces into the subcompartments collar, lip, and basal ring (Strausfeld et al., 1998). It is possible that the relation between learning and the olfactory region of the calyces, the lip, is observable at the level of volume analysis, but not observable for the visual region, the collar.

Another possible explanation for the absence of a relation between mushroom body volume and learning or reversal learning is that there was insufficient variation in the sample of bumblebees used here. In bumblebees, larger bees are more likely to become foragers while smaller bees remain in the colony and engage in other tasks (Goulson, 2003). Consequently foraging bumblebees represent only a limited range of the body sizes present within the colony. All of the bees used here were foragers resulting in a restricted range on bumblebee body sizes in the sample. Brain volume and mushroom body volume correlate with body size in bumblebees

(Mares et al., 2005), so using a sampling technique that restricted the body size of bumblebees likely resulted in a restricted the range of bumblebee mushroom body volumes. There was, however, considerable variation in mushroom body volume among the bees in the present study and strong relations between mushroom body components and total mushroom body volume (Figure 4-11) and between calyx volume and calyx subcompartments (Figure 4-12). Similarly, there was considerable variation in bees' ability to learn the initial discrimination and perform reversal learning (Figure 4-4). It is therefore unlikely that the absence of correlations between mushroom body volume and learning were due to lack of sensitivity in the behavioural and anatomical measures used.

All bees observed were experienced foragers. It has been demonstrated repeatedly in honeybees (Withers et al., 1993; Withers et al., 1995) and also in bumblebees (Riveros & Gronenberg, 2010) that the mushroom bodies undergo experience-related volume changes with the onset of foraging and acquisition of foraging experience. By using only foraging bees, my sample may have been restricted to bees that have already undergone foraging-related brain changes. For this study the inclusion criterion of foraging experience was necessary because smaller or inexperienced bees would not have been able to complete the behavioural task. It may be possible, however, to examine learning and reversal in a broader range of bumblebees with a different behavioural technique.

A consequence of the absence of any relations between brain measurements and of the behavioural measures is that the data provide no information regarding the two competing hypotheses surrounding behavioural flexibility in bumblebees. The first hypothesis is that flexibility in reversal learning is the consequence of a breakdown in associative learning, implying a negative correlation between mushroom body function and flexibility. The second

hypothesis, proposed by Raine and Chittka (2012), is that a common mechanism underlies both learning and flexibility, predicting that positive correlations would exist between both discrimination learning and mushroom body function and between reversal learning and mushroom body function. Investigations of these hypotheses will require more detailed characterization of the mushroom bodies such as synaptic organization (Hourcade et al., 2010; Li et al., 2017).

4.4.2 Bumblebee mushroom bodies

The honeybee brain has been studied more extensively than the bumblebee brain, producing a number of volume estimates of mushroom body subcompartments (Durst et al., 1994; Withers et al., 1993; Withers et al., 1995). Extensive investigations into the developmental changes that occur in the mushroom bodies of honeybees has produced volume measurements for all component parts of the mushroom bodies in honeybees, including the subcompartments of the calyces (Durst et al., 1994; Withers et al., 1993; Withers et al., 1995). Although measurements of bumblebee mushroom body volume have been published (Mare et al., 2005; Riveros & Gronenberg, 2010), analysis of the mushroom body subcompartments has not. In addition to consideration of the relation between behavioural flexibility and the mushroom bodies, this study provides the first volume measurements of the subcompartments of the bumblebee mushroom body calyces.

All structures within the mushroom bodies of the bumblebee varied allometrically with overall mushroom body volume, that is, each of the internal structures of the mushroom bodies was larger in mushroom bodies that had a larger total volume. Of the correlations between subdivisions in the mushroom bodies two are of particular interest. First, volume of the peduncle and lobes did not correlate with calyx volume, and second, the largest structure within the

mushroom bodies was the collar subcompartment within the calyces. The latter is particularly interesting because this contrasts with results for foraging honeybees, in which the peduncle and lobes are the largest contributor to total mushroom body volume (Durst et al., 1994). The percentage of total mushroom body volume attributable to the lip and basal ring subcompartments within the calyces was comparable to foraging honeybees (Durst et al., 1994), suggesting that the bumblebees have greater relative investment specifically in the collar, the visual processing region of the calyces. Once again, it is important to consider these findings in light of the bumblebee sample used here. The lobes of larger bumblebees tend to have a smaller relative volume than they do in smaller bumblebees, but this is not found in the calyces, which maintain the same relative size in larger bumblebees as in smaller bumblebees (Mares et al., 2005). The sampling of exclusively larger bumblebees in this study may have resulted in data representing the most extreme investment in calyx volume relative to the lobes, and the relative contribution of the subdivisions may not hold across the full range of bumblebee body sizes. Despite the limits of the sample here, these data do suggest a difference in investment in the visual subcompartments of the calyces between honeybees and bumblebees.

4.4.3 Conclusions

The primary goal of this chapter was to explore the relation between behavioural flexibility and the mushroom bodies of the bumblebee brain. Correlations between reversal learning and volume of the mushroom bodies, and specific structures within the mushroom bodies, showed none of the predicted relations. Prior research indicating a relation between the mushroom bodies and learning suggests that the absence of significant correlations in the present study does not necessarily indicate the absence of a relation between the mushroom bodies and reversal learning, but rather that learning and mushroom body volume are not directly correlated.

A second goal of this chapter was to fully describe the volume of subdivisions within the bumblebee mushroom bodies. This was done successfully and the resulting measurements produced a more complete characterization of the bumblebee mushroom bodies.

4.5 References

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Chapter 5

5 General Discussion

The research described in this thesis was designed to explore behavioural flexibility in bumblebees (*Bombus impatiens*). This was done through a series of experiments on flower handling and an experiment on how variation in flexibility is related to variation in mushroom body volume.

Chapter 2 described the development of a model of flower handling behaviour that could be used under controlled laboratory conditions and made it possible to quantify flower handling learning with greater specificity than has been previously accomplished. The model was successful and provided data showing two components to the acquisition of flower handling techniques: (1) innate motor patterns, and (2) the increase in use of the successful motor pattern by reinforcement and operant learning. These findings demonstrate that bumblebees generate species level flexibility through the efficient use of both innate processes and simple learning mechanisms. Outlining the mechanisms of flower handling learning with this degree of specificity compliments the extensive descriptions of flower handling in the wild provided by Heinrich (1976a; 1976b) and Laverty (1980), and contributes to our understanding of how the simple nervous systems of bees can generate complex flexible output (Giurfa, 2003).

The model developed in Chapter 2 was adapted to address outstanding questions about Darwin's interference hypothesis (Darwin, 1876). Darwin suggested there should be efficiency costs when bumblebees switch between flower species while foraging and consequent inflexibility in foraging behaviour at the level of the individual. It has previously been demonstrated that bumblebees do not show the expected efficiency costs at an ecologically relevant level (Woodward & Laverty, 1992), but the mechanism by which they avoided these

costs was not fully explored. My experiments show that when bees switch between two flower types they switch between two innate motor patterns rather than investing in learning two novel motor patterns. The absence of learning at the motor pattern level eliminates the possibility of memory interference in motor patterns and minimizes the occurrence of interference in flower handling overall. This probably explains the presence of minor interference costs, due to changes in the use of motor patterns, and why these interference costs are ecologically inconsequential.

Previous research has shown a relation between mushroom body volume and discrimination learning in honeybees (Gronenberg & Couvillon, 2010). The relation between mushroom body volume, learning, and behavioural flexibility in bumblebees was explored in Chapter 4. The experiment involved measuring bumblebee behavioural performance on a reversal task, a common measure of behavioural flexibility (Audet & Lefebvre, 2017), and relating that performance to detailed volume analysis of bumblebee mushroom bodies. There was no relationship between behavioural measures of learning and flexibility and mushroom body volume in foraging bumblebees. Despite finding no significant relations between mushroom body volume and learning, the extensive characterization of mushroom body volume provided in this chapter provides original new information on the structure of the bumblebee mushroom bodies.

5.1 Behavioural flexibility

Aside from the specific goal of characterizing behavioural flexibility in bumblebees, the experiments in this thesis had the broader goal of exploring the cognitive mechanisms that generate complex behaviour. The field of animal cognition routinely experiences cycles which begin with the description of an exciting ‘human like’ cognitive process in an animal and then following more rigorous experimentation the revelation of simpler learning mechanisms that are

actually involved (Shettleworth, 2010). Studies of "insight" have shown this cycle multiple times in the history of animal cognition. The first was Köhler's (1959) studies with chimpanzees which showed them generating the completely novel solution of stacking boxes to access a banana that was out of reach. The chimpanzees' behaviour was proposed to be akin to the 'aha' moment that humans experience during problem solving by insight. The subsequent demonstration of a pigeon accomplishing the same feat as the chimpanzees and generating a novel solution after reinforcement and punishment of a few key behaviours directed to the boxes, provided a simpler explanation for the behaviour (Epstein et al., 1984). The chimpanzees probably had very similar reinforcement experience with their boxes prior to their "insightful" solution. More recently, problem solving by insight was described in corvids by Bird & Emery (2009), but further experiments showed that previous experience, not "insight" was the critical factor in the birds' problem solving (von Bayern et al., 2009).

Studies of behavioural flexibility, particularly those with bees, are likely at a turning point in which exciting and unexpected behaviour has been described and now steps will be taken to understand the cognitive mechanisms responsible. The recent publication of papers critical of the methods used to study behavioural flexibility (Audet & Lefebvre, 2017; Mikhalevich, Powell, & Logan, 2017) suggest this is the case. There is research to support a relation between behavioural flexibility and complex cognition (Lefebvre, Reader, & Sol, 2004; Reader, Hager, & Laland, 2011), but foundational principles of the field such as Lloyd Morgan's Canon (1903) remain important. Seemingly complex behaviour can be the outcome of simpler cognitive processes.

The experiments described in Chapters 2 and 3 demonstrate a broadly applicable approach to studying behavioural flexibility. This included the development of an apparatus with ecological

relevance to the study species, rather than a puzzle box (Auersperg, Gajdon, & von Bayern, 2012) or an intentionally novel task (Mirwan & Kevan, 2014; Loukola et al., 2017), and quantification of behaviour in greater detail than success or failure. Some recent studies of bumblebee problem solving and behavioural flexibility have provided interesting examples of the capabilities of bees but their application to understanding bee behaviour has been limited by the at times intentional absence of ecological relevance. In some tasks bees undergo extensive operant training before they can successfully manipulate the apparatus used (Mirwan & Kevan, 2014; Alem et al., 2016; Loukola et al., 2017). Research of this kind excludes one of the most intriguing aspects of object manipulation or flower handling by bees in the wild which is their ability to acquire behaviour in the absence of reward (Muth, Keasar, & Dornhaus, 2015).

In this thesis I explored behavioural flexibility using behaviour that naturally occurs in flower handling. Bumblebees have a large repertoire, however, of other behaviours that researchers can use to continue asking questions about problem solving and flexibility. A perfect example is nectar robbing, or as Darwin described it "the felonious practice of biting holes through the corolla"(Darwin, 1976, p.420). The behavior of chewing a hole in the corolla of difficult to handle flowers in order to reach the floral nectary seems very similar to the 'opening a window' solution of the Multi Access Box developed by Auersperg et al. (2012), making it a further naturally occurring behaviour with which to study problem solving in bees.

5.2 Contributions to bumblebee neuroscience

There is a great deal of previous research on mushroom body volume in honeybees because they were the bee species of choice during a boom in honeybee neuroscience in the late 1990s and early 2000s that focussed largely on the volume of brain structures (Withers, Fahrbach, & Robinson, 1993; Durst, Eichmüller, & Mezel, 1994; Withers, Fahrbach, & Robinson, 1995).

Bumblebees are increasingly being used in neuroscience work (Riveros & Gronenberg, 2009), but without analysis of the size of brain structures. Instead bumblebee brains are investigated at the level of synaptic organization (Li et al., 2017). Progress in bee neuroscience has left us without much basic knowledge of the bumblebee brain. In Chapter 4, I provided new data on the mushroom bodies of the bumblebee brain and their component parts. Future comparative work on the mushroom bodies of the honeybee and bumblebee brain would be particularly interesting in light of differences in cognition and behaviour between these two important pollinators (Sherry & Strang, 2015)

The failure to find a relation between mushroom body volume and learning or behavioural flexibility is an interesting observation given previous findings in honeybees (Gronenberg Couvillon, 2010). It supports the point that there are potential differences between honeybees and bumblebees in mushroom body volume that are underexplored. The absence of correlations between mushroom body volume and performance suggests, however, that further investigation of the relationship between the brain and behaviour in bumblebees at a finer level of analysis, such as synaptic organization (Li et al., 2017) is likely to be more productive.

5.3 Implications for conservation

Given the current declines in some bumblebee populations (Cameron et al., 2011) the application of basic research to conservation is an important consideration in the study of bumblebees and was certainly a consideration in this thesis. One of the main threats to pollinators are pesticides, which even when not applied at lethal levels can have sublethal effects on colony and population survival (Whitehorn et al., 2012; Phelps et al., 2018). The pervasiveness of pesticide exposure in the wild (Bonmatin et al., 2015) makes it challenging to find unexposed bee populations in the wild, making it challenging to conduct controlled studies

on the effects of pesticides. The flower handling task I developed can be used to assess the sublethal effects of pesticides on foraging behaviour in a controlled laboratory setting while maintaining ecological relevance. Both motor and learning deficits can be observed on this task given that both innate motor patterns and learning are required to solve it. My flower handling model can be used in conjunction with pesticide treatment, or the application of other stressors, to examine the effects of human-induced rapid environmental change on bee populations.

5.4 Conclusions

Bumblebees are important in the study of animal cognition because of their contributions to our food supply and economy (Potts et al.,2010), and their accessibility as a model for cognitive and neural processes (Riveros & Gronenberg, 2009). In this thesis, I explored one of the most remarkable features of bumblebees, their ability to generate complexity out of simplicity. This feature has made them useful for exploring cognition in general, and an ideal model for exploring behavioural flexibility. My findings on behavioural flexibility in bees show that general conclusions about the relationship between behavioural flexibility and intelligence or complex cognition need to be made with caution and consideration of mechanisms. When discussing the evolution of "endless forms" from "so simple a beginning" in the concluding lines of On the Origin of Species, Darwin wrote "there is grandeur to be found in this view of life" (Darwin, 1859). Darwin's sentiment is directly applicable studies of cognition in bumblebees. There is grandeur to be found in understanding how a seemingly simple organism can produce complex behaviour in the form of flower handling and pollination that has an outcome of global importance.

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von Bayern, A. M., Heathcote, R. J., Rutz, C., & Kacelnik, A. (2009). The role of experience in problem solving and innovative tool use in crows. *Current Biology*, *19*(22), 1965-1968.

von Frisch, K. (1967). The Dance Language and Orientation of Bees. Cambridge: Harvard University Press

Whitehorn, P. R., O'Connor, S., Wackers, F. L., & Goulson, D. (2012). Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science*, 1215025.

Withers, G. S., Fahrbach, S. E. , & Robinson, G. E. (1993). Selective neuroanatomical plasticity and division of labour in the honeybee. *Nature, 364,* 238-240.

Withers, G. S., Fahrbach, S. E., & Robinson, G. E. (1995). Effects of experience and juvenile hormone on the organization of the mushroom bodies of honey bees. *Journal of Neurobiology, 26,* 130-144.

Woodward, G. L., & Laverty, T. M. (1992). Recall of flower handling skills by bumble bees: A test of Darwin's interference hypothesis. *Animal Behaviour, 44*, 1045-1051.

Curriculum Vitae
Caroline Galloway Strang

Academic History

Ph.D. Psychology 2011- Present
Department of Psychology, University of Western Ontario
Advisor: Dr. David Sherry

Masters of Science – Psychology 2009 – 2011
Department of Psychology, University of Western Ontario
Advisor: Dr. David Sherry

Honours Bachelor of Science – Psychology 2003 - 2009
Department of Psychology, University of Toronto

Academic Exchange – Non-Graduating Student 2005 – 2006
University of St. Andrews, St. Andrews, Scotland

Research Experience

Doctoral Research 2011 – present
Department of Psychology
University of Western Ontario

Visiting Scholar - Universidade Federal do Pará January 2012

Masters Research 2009 – 20011
Department of Psychology
University of Western Ontario

Volunteer – Affect and Cognition Laboratory Summer 2009
University of Toronto
Supervisor: Dr. Eve De Rosa

Individual Research Project Fall 2008
University of Toronto
Advisor: Dr. Eve De Rosa

Individual Research Project Summer 2008
University of Toronto
Advisor: Dr. Eve De Rosa

Volunteer – Affect and Cognition Laboratory Spring 2008
University of Toronto
Supervisor: Dr. Eve De Rosa

Awards and Scholarships

G. Keith Humphrey Memorial Award 2016

Ontario Graduate Scholarship 2014-2015

Ontario Graduate Scholarship 2013-2014

2013 Three Minute Thesis (3MT) Competition – Runner-up 2013
University of Western Ontario

2012 Three Minute Thesis (3MT) Competition - Finalist 2012
University of Western Ontario

Graduate Thesis Research Award 2011-2012
University of Western Ontario

Committees

Conference on Comparative Cognition (CO3) Apr 2018
Team CO3

Conference on Comparative Cognition (CO3) Apr 2017
Team CO3

UWO Department of Psychology Space and Facilities Committee 2015-2016
Committee Member

Colloquium Organizing Committee 2014-2015
Committee Member

Ontario Ecology, Ethology, and Evolution Colloquium 2013 2012-2013
Organizing Committee Member

Psychology Graduate Students' Association 2010-2011
Executive Committee Member

Editorial Duties

Guest co-editor
Learning and Motivation Special Issue on Insect Cognition (2015, v50)
Reviewer
Journal of Insect Behavior
Learning and Motivation
Animal Cognition

Teaching Experience

Part Time Faculty at Western University in London, ON.

- Evolution and Psychology: The science of human nature (Psychol 3228), Fall 2017
- Evolution and Psychology: The science of human nature (Psychol 3228, online), Summer 2017
- Introduction to Animal Cognition (Psychol 2210), Winter 2017
- Introduction to Behavioural and Cognition Neuroscience (Psychol 2220, online), Fall 2015

Contract Faculty at Brescia University College in London, ON.

- Research Methods II (Psychol 2856), Winter 2018
- Research Methods I (Psychol 2855), Winter 2018
- Research Methods II (Psychol 2856), Winter 2017
- Drugs & Behaviour (Psychol 2020), Fall 2016
- Motivation and Emotion (Psychol 3209), Winter 2016
- Drugs & Behaviour (Psychol 2020), Fall 2015.

Graduate Teaching Assistant for the following courses at Western University in London, ON.

- Introduction to Animal Cognition (2210), Winter 2015 and Winter 2014
- Animal Behaviour (3221), Fall 2014, Fall 2013, and Fall 2012
- Psychology of Persuasion (3721), Winter 2013
- Evolution and Human Behaviour (3229), Winter 2011
- Hormones and Behaviour (3226), Winter 2011
- Research Methods and Statistical Analysis in Psychology (2820), Fall 2011
- Neuroscience of Motivation and Emotion (3209), Winter 2010

Supervision of Undergraduate Honours Theses

- M. Boivin (Co-supervised with D. F. Sherry) 2017-18
- J. Phelps (Co-supervised with D. F. Sherry) 2015-16
- S. Noyek (Co-supervised with D. F. Sherry) 2015-16
- A. Ngo (Co-supervised with D. F. Sherry) 2011-12

Invited Talks

Strang, C. G. (2018, November). Animal minds: Insights on animal cognition from Fifteen Dogs. A public lecture in recognition of Fifteen Dogs as part of the Algoma Reads Initiative.
Algoma University, Sault Sainte Marie, Ontario

Strang, C. G. (2012, January). Behavioural flexibility in bumblebees. Invited talk – Instituto Federal De Educação, Ciência e Tecnologia, Pará, Brazil

Strang, C. G. (2012, January). Behavioural flexibility in bumblebees. Invited talk – Universidade Federal do Pará, Pará, Brazil

Guest Lectures

Strang, C. G. (2015, February). Social insects. Guest lecture – Psychology 2210 Animal Cognition. University of Western Ontario.
London, ON.

Strang, C. G. (2014, December). Social insects. Guest lecture – Psychology 3221 Animal Behaviour. University of Western Ontario.
London, ON.

Strang, C. G. (2011, April). Behavioural flexibility in bumblebees. Guest lecture – Psychology 3226 Hormones and Behaviour, University of Western Ontario, London, ON.

Strang, C. G. (2010, March). What can bumblebees do? Guest Lecture – Psychology 3209 Neuroscience of Motivation and Emotion, University of Western Ontario, London, ON.

Publications

Phelps, J. D., **Strang, C. G.**, Gblik-Sikorska, M., Sniegocki, T., Posyniak, A., & Sherry, D. F. (2018). Imidacloprid slows the development of preference for rewarding food sources in bumblebees (Bombus impatiens). *Ecotoxicology, 27 (2)*, 175-187.

Guitar, N. A, **Strang, C. G**., Course, C. J., & Sherry, D. F. (2017). Chickadees neither win-stay nor win-shift when foraging. *Animal Behaviour, 133*, 73-82.

Roberts, W. A., Macpherson, K., & **Strang, C. G.** (2016). Context controls access to working and reference memory in the pigeon(*Columba livia*). *Journal of the Experimental Analysis of Behavior, 105*, 184-193.

Roberts, W. A., **Strang, C. G.**, & Macpherson, K. (2015). Memory systems interaction in the pigeon: Working and reference memory. *Journal of Experimental Psychology: Animal Learning and Cognition*, *41*, 152-162.

Sherry, D. F. & **Strang, C. G.** (2015). Contrasting styles in cognition and behaviour in bumblebees and honeybees. *Behavioural Processes*, *117*, 59-69.

Strang, C. G. & Sherry, D.F. **(**2014). Serial reversal learning in bumblebees (*Bombus impatiens*). *Animal Cognition,17*, 723-734.

Conference Papers **(* indicates presenting author)**

Strang, C. G.*, & Sherry, D. F. (2015, April). Problem solving in bumblebees. Conference on Comparative Cognition. Melbourne, Florida.

Strang, C. G.*, & Sherry, D. F. (2014, May) Problem solving in bumblebees. Ontario Ecology, Ethology, and Evolution Colloquium. Guelph, ON.

Roberts, W. A., **Strang, C. G.***, & Macpherson, K.* (2014 March). The interaction of working and reference memory in pigeons. Conference on Comparative Cognition. Melbourne, Florida.

Strang, C.G.*, & Sherry, D. F. (2012, May). Behavioural flexibility in bumblebee (Bombus impatiens) reversal learning and the mushroom bodies. Ontario Ecology, Ethology, and Evolution Colloquium. McMaster University, Hamilton, ON.

Strang, C. G.*, & Sherry, D. F. (2011, October). Brain and behaviour in bumblebees (*Bombus impatiens*): Reversal learning and the mushroom bodies. *2nd Annual Biology Graduate Research Forum*, University of Western Ontario. London, ON.

Conference Posters (* indicates presenting author)

Roberts, W. A.*, **Strang, C. G.**, & Marsh, H. (2015, April). Do rats prefer informative over non-informative stimuli? Evidence from a radial arm maze. Conference on Comparative Cognition. Melbourne Florida.

Strang, C. G.*, & Sherry, D. F. (2014, March) Spatial working memory in horses. Conference on Comparative Cognition. Melbourne, Florida.

Strang, C. G.*, & Sherry, D. F. (2014, March). Spatial working memory in horses. Mechanisms of Learning Forum 2014. Atlanta, USA.

Strang, C. G.*, & Sherry, D. F. (2013, August). Spatial cognition in horses. *Behaviour 2013 – International Ethological Conference and meeting of the Association for the Study of Animal Behaviour*. Newcastle, UK.

Strang, C. G.*, Ngo, A. P., & Sherry, D. F. (2012, October). The effects of maturation and foraging experience on behavioural flexibility and the mushroom bodies of bumblebees (Bombus impatiens). *Neuroscience 2012 – Annual Meeting of the Society for Neuroscience*. New Orleans, USA.

Strang, C. G.*, & Sherry, D. F. (2012, July). Visual attention and the mushroom bodies in bumblebees, Bombus impatiens. *8th FENS Forum on Neuroscience*, Barcelona, Spain.

Strang, C. G.*, & Sherry, D. F. (2011, August). Learning flexibility in bumblebees. *The Association for the Study of Animal Behaviour Summer Conference*. St. Andrews, Scotland

Strang, C. G.*, & Sherry, D. F. (2011, March). Serial reversal learning in bumblebees. *18th Annual International Conference on Comparative Cognition. Melbourne*, Florida.

Strang, C. G.*, Botly, L. C. P., & De Rosa, E. (2009, March). Probing inhibitory control in rats. *16th Annual International Conference on Comparative Cognition. Melbourne*, Florida.

Media Coverage

Gradcast: Our Partners in Science with Caroline Strang (e14), April 22, 2015

Londoner, June 3, 2014. *Flight of the Bumblebee* by Don Biggs

The Cardinal (Nature London), No. 237, November, 2014. *Research at Western: Discoveries of The Black-Capped Chickadee* by Leslie Kostal